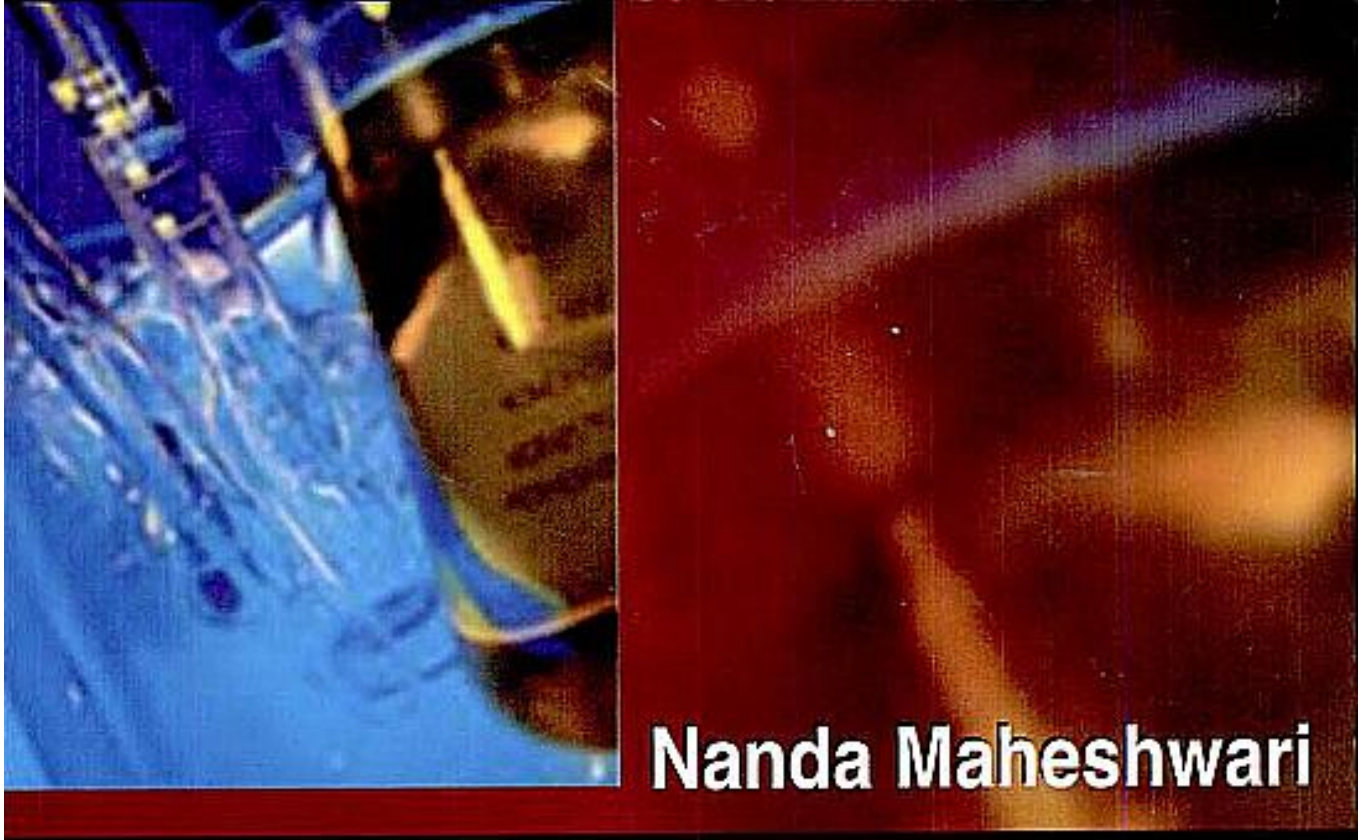

Nanda Maheshwari

Clinical Biochemistry

Foreword
Rajesh Karajgaonkar

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Clinical Biochemistry

Clinical Biochemistry

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Foreword

There is a great need of good books on Clinical Biochemistry for CMLT/DMLT and undergraduate courses. This book, written by Nanda Maheshwari, is a very appreciable effort to meet the requirements of students.

It covers all the required topics in a descriptive and synoptic style. Simple language and up-to-date data used by the author makes it easy for the student to understand the concept.

I congratulate the author for this third book after her two earlier titles—Clinical Pathology and Haematology and Clinical Microbiology. May Almighty give her courage and achievements she deserves. I wish her great success.

Rajesh Karajgaonkar
Director, Institute of Paramedical Science
Nanded, Maharashtra

Preface

It is a matter of great pleasure to present my third book *Clinical Biochemistry*. In this book, I have tried to explain various concepts using simple language and short sentences. Each topic covered in one small chapter makes it easy for the student to read. The aims and objects of this book are primarily to meet the requirements of CMLT/DMLT and undergraduate courses.

Clinical Biochemistry is an important subject of Medical Laboratory Technology courses, but there is non-availability of good books on this subject. The books available are vast and mainly written for MBBS students. I have tried to overcome this problem by writing in easy language and providing exact data. I hope that this book will satisfy the needs of students.

I invite and welcome constructive criticism and suggestions from teachers, colleagues and students for next revised edition.

Nanda Maheshwari

Acknowledgements

First of all I would give my sweetest regards to my little angel Arushi for understanding my business and being patient like a mature person, during my work on this title.

I am indebted to Mr. Rajesh Karajgoanker for inspiring me to write this book and the confidence shown towards me. He gave me facility and encouragement during the course of writing. My sincere thanks to my mother Kusumlata, who has always encouraged and supported my efforts. I would like to place on record my gratitude towards my parents, in-laws, students, colleagues, friends and superiors from whom I received help and support for writing the book.

I specially want to thank my friend Neeta Mundada who has devoted very careful efforts and time to do valuable corrections in the script. My husband Yogendra's contribution in making of this book can't be put in words, which has encouraged me at every step in the making of the book.

Finally, I would like to thank M/s. Jaypee Brothers Medical Publishers (P) Ltd. New Delhi, who have decided to publish the book.

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Part I
Biochemistry

1 Elementary Knowledge of Inorganic Chemistry

STRUCTURE OF ATOM

All matter is composed of minute discrete particles called as atoms. An atom is composed of still smaller particles viz. electrons, protons and neutrons (Fig. 1.1). These are known as atomic or fundamental particles of an atom. The proton is a positively charged particle situated in highly dense central part of an atom called nucleus. The electron is a negatively charged particle and is present in the extra nuclear part of an atom. The neutron is a neutral particle present in the nucleus of the atom.

As the electrons have negligible weight, the mass of the atom is concentrated at the nucleus. The number of electrons present in the extra nuclear part is equal to the number of protons present in the nucleus. Thus, an atom is electrically neutral.

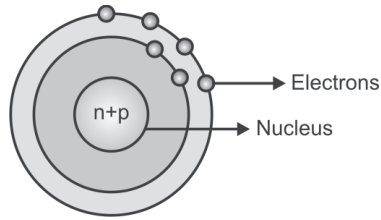


Fig. 1.1: Structure of atom

Atomic Weight

Since an atom is very tiny, it is inconvenient to calculate the absolute weight of an atom. Therefore, weight of the atom of an element is compared to the weight of the atom of a standard element like hydrogen or oxygen.

Atomic weight of an element is defined as the average relative weight of its atom as compared with $\frac{1}{12}$th weight of one atom of carbon isotope of mass 12, e.g.

Element	*Atomic weight*
Mg	24.32
Al	26.98
Cu	63.54

Molecular Weight

Like atoms, molecules are also very small; hence molecular weights like atomic weights are also relative. Molecular weight of a substance is defined as the average relative weight of its molecule compared with the $\frac{1}{12}$th weight of one atom of carbon isotope of mass 12. Molecular weight of a substance is also the sum of atomic weights of all atoms present in its molecule.

e.g. 1. Methane (CH_4) = 12+4 = 16

2. Sulfuric acid H_2SO_4 = 98

Equivalent Weight

Whenever an element takes part in a chemical reaction, it must be associated with a definite weight or some multiple of that definite weight.

Equivalent weight of an element is that weight of it, which can combine with or replace from a chemical combination 8 parts by weight of oxygen or 35.5 parts by weight of chlorine or 1.008 parts by weight of hydrogen.

It is also defined as the weight of an element, which liberates 11.2 liters of pure and dry hydrogen gas at N.T.P. when the equivalent is expressed in grams, e.g.

Element	*Equivalent weight*
Al	9
Sn	29.75
O_2	12.3

ACIDS, BASES AND SALTS

The term acid is derived from a Latin word acidus means sour; while the term alkali is originated from an Arabic word alkali means plant ash.

According to the classical idea, acid is a substance whose water solution —

i. Turns blue litmus to red.
ii. Neutralize base.
iii. Reacts with active metals with the evolution of hydrogen gas.
iv. Has sour taste and
v. Decomposes carbonates into CO_2 and H_2O.

According to the classical idea, base is a substance whose water solution —

i. Turns red litmus to blue.
ii. Neutralize acid.
iii. Has bitter taste.
iv. Feels soapy and
v. Absorbs CO_2 to form carbonate.

These definitions were applicable to aqueous solution and also do not mention their structures. Therefore, different modern concepts were developed to define acids and bases.

Arrhenius Theory of Acids and Bases (Water-ion-concept)

According to Arrhenius (1887) an acid is a hydrogen compound, which in aqueous solution gives hydrogen ions.

e.g. $HCl \rightarrow H^+ + Cl^-$

A base is a hydroxide compound, which in aqueous solution produces hydroxyl ions.

e.g. $NaOH \rightarrow Na^+ + OH^-$

Thus, acidic properties are due to H^+ and basic properties are due to OH^- ions.

Lowry and Bronsted Concept of Acids and Bases (Protonic Concept)

According to Lowry and Bronsted (1923) an acid is a substance, which can donate a proton (H^+ ion) i.e, acid is a proton donor.

Base is a substance, which can accept a proton (H^+ ion) i.e., base is a proton acceptor.

e.g. $HCl + H_2O \rightarrow H_3O^+ + Cl^-$

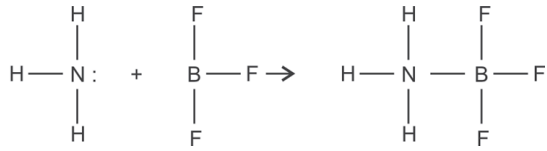
Here, since HCl donates a proton to water it is an acid. Water accepts a proton, hence it is a base.

Lewis Concept of Acids and Bases (Lewis Electronic Theory)

According to Lewis (1923) an acid is a substance which can accept the electron pair to form a co-ordinate bond. Thus, acid is an electron acceptor.

Base is a substance, which can donate the electron pair to form a co-ordinate bond. Thus, base is an electron donor.

e.g. $NH_3 + BF_3 \rightarrow NH_3\,BF_3$



Ammonia + Boron trifluoride → co-ordinate complex

In above reaction, BF_3 is a Lewis acid and is accepting an electron pair and NH_3 is a Lewis base as it is donating an electron pair.

SALTS

Salt is a substance of which cation or anion or both the ion reacts with water producing acidity or basicity in the solution. OR

A salt is a compound formed by the interaction of an acid and a base, placing H^+ of the acid by a metal or metal like radical.

Neutralization reaction of acids and base forms salt and water.

e.g. $HCl + NaOH \rightarrow NaCl + H_2O$

acid + base salt + water

Types of Salts

Depending on the behavior of salts in aqueous solution, there are four types of salts —

1. Salt of strong acid and strong base: These salts produce strong acid and strong base when react with water.
   e.g. KCl, NaCl, KNO_3, Na_2SO_4
2. Salt of strong acid and weak base: These salts produce strong acid and weak base when react with water.
   e.g. NH_4Cl, $CuSO_4$, $FeCl_3$
3. Salt of weak acid and strong base: These salts produce weak acid and strong base when react with water.
   e.g. KCN, NaCN, CH_3COONa
4. Salt of weak acid and weak base: These salts produce weak acid and weak base when react with water.
   e.g. CH_3COONH_4, $(NH_4)CO_3$

The cell contains 1 to 3% salts.

Sodium chloride is a common salt that is added to prepare food. It regulates plasma volume, acid-base balance and nerve as well as muscle function. It maintains fluid and electrolyte balance. Chloride of salt is a source of HCl in gastric juice. It also acts as enzyme activator.

The other important mineral salts present in cells and animal body are –

H^+, K^+, Ca^{++}, Mg^{++}, Cl^-, OH^-, HCO^-_3,$CO^-_{3,}SO^-_4$,PO^-_4

The Salts Serve Following Functions

1. Salts are essential for construction, survival and growth of cells.
2. The metallic ions function as catalysts of enzymes.
3. Ions maintain electrical properties of the cells.
   e.g. Na^+ and K^+ ions are necessary for nerves to conduct impulses.
4. Ions act as buffers. e.g. HCO_3 in blood.
5. Ions maintain atmospheric pressure.
6. Ions are used for synthesis of tissues.
   e.g. iron is used for synthesis of tissue, calcium is used for synthesis of bones.

pH INDICATORS, pH METER, pH MEASUREMENT

pH Indicators

A number of organic substances are known which show distinctly different colours below and above a small pH range.

e.g. Phenolphthalein is colourless below a pH of 8.3 and distinctly pink above 10. Over the pH range 8 to 10 it changes colour gradually through different shades of pink. Methyl orange shows a distinct red colour below pH 3.1 and a yellow colour above a pH of 4.4.

Colour Change

Indicator	*Acid*	*Alkali*	*pH range*
Thymol blue	Red	Yellow	1.2-2.8
Methyl yellow	Red	Yellow	2.9-4.0
Methyl orange	Red	Yellow	3.1-4.4
Methyl red	Red	Yellow	4.2-6.3
Bromothymol blue	Yellow	Blue	6.0-7.6
Phenol red	Yellow	Red	6.8-8.4
Cresol red	Yellow	Red	7.2-8.8
Phenolphthalein	Colourless	Pink	8.3-10.0

For quickness and convenience, indicator papers are made from universal indicator are available. From the colour developed with the universal indicator, one can easily fix the approximate value of the pH of a solution. By comparing the colour developed with the already prepared standardized colour plates or charts, the pH of a solution under test can be fixed with a remarkable accuracy.

pH Meter

The pH meter is used to measure the pH of a solution.

The pH meter is of two types—Digital pH meter and manual pH meter.

pH meter consists of power pack and two electrodes.

The power pack contains an on/off switch, an indicating meter, a temperature compensation knob, a calibrate knob, and a wire with a plug pin. The on/off switch is used to supply or cut off electric current. The indicating meter shows pH reading. The temperature compensation knob is used to adjust the temperature. The calibrate knob is used to set pH. The plug pin is connected to the main electric line.

The pH meter contains two electrodes—glass electrode and a calomel electrode. In modern pH meters the two electrodes are combined into single unit.

The glass electrode has a hard glass tube. At the base, it has a thin bulb. The bulb contains HCl (0.1 mol/lit.). The bulb is covered by a special membrane of soda glass. It is sensitive to H^+ and it allows H^+ to pass through it. A platinum wire is connected to the HCl through silver – silver chloride electrode. The wire coming out from the glass electrode is connected to the power pack of the pH meter.

The calomel electrode is a reference electrode. It is not sensitive to H^+. It contains a calomel paste. The calomel paste is connected with a platinum wire through mercury. The free end of the calomel electrode has a porous plug. The base of electrode is deposited with KCl crystal. The remaining portion is filled with KCl solution.

The glass electrode in the test solution constitutes a half-cell and the calomel electrode constitutes the other half-cell. The two electrodes complete the circuit.

Principle: If the conductor is immersed into an appropriate electrolyte solution there will be a tendency for its atoms to leave the surface and enter the solution as ions. This is called a half-cell and the conductor is called an electrode. The potential difference between the two half-cell is called the electromotive force. (emf). The emf will change if the flow of electrons is changed.

This is what happens in the measurement of pH. The indicator electrode is dipped in a solution whose hydrogen ion concentration is to be measured. H^+ ions are drawn towards the outer surface of the glass membrane. The number of H^+ ions accumulating on the membrane depends on their concentration in the external solution.

The change in the hydrogen ion activity outside the glass bulb changes the potential between the indicator electrode and the calomel electrode. The potential is measured by sensitive voltmeter.

The instrument must be calibrated against buffers of known pH before measuring the pH of unknown specimen.

Procedure and Precaution of Measurement of Blood pH

pH of blood at body temperature (37°C) is different than at room temperature. Therefore, the measurement should be made at 37°C and sample should not be exposed to atmosphere. Only heparinised plasma should be used for measurement of blood pH.

Following steps should be followed for pH measurement of blood:

i. Turn on the pH meter at least 15 min prior to use. Calibrate the electrode using a standard buffer at 37°C making sure to select the proper pH of buffer at 37°C and to set temperature of pH meter at 37°C. The recommended buffers for plasma pH calibration are 7.384 and 8.841.
ii. Blood samples must be kept anaerobically to prevent loss of absorption of CO_2. The pH measurement should be made within 15 min after sample collection or sample should be kept on ice and measurement should be made within 2 hrs. The samples should be equilibrated to 37°C before measurement.
iii. To prevent the coating of electrode the sample from the electrode should be flushed with saline solution after each measurement. A residual blood film can be removed by dipping for a few min in 0.1 M NaOH followed by 0.1 M HCl and water or saline.

It is a common practice to take venous blood for pH measurement. pH range for arterial blood is — 7.31 to 7.45. Venous blood may differ from common arterial blood by up to 0.03 pH unit.

SOLUTIONS

Solution is a combination of solute and solvent. A solute dissolves in solvent and the preparation is called as solution.
Solute + Solvent = Solution

Basically Solutions are of two Types:

1. Saturated solution and
2. Standard solution.

1. **Saturated solution:** In the saturated solution neither weight of solute is not specified nor is the volume of solvent. Thus, any volume of solvent in which maximum amount of solute is dissolved is saturated solution. The strength of solution is not known here.
2. **Standard solution:** In the standard solution weight of solute and volume of solvent both are known. Thus, it is the preparation made from known volume of solvent and known weight of solute. The strength of solution can be determined here.

Strength of solution can be expressed in four ways.

1. Percent solution
2. Part dilutions
3. Molar solution
4. Normal solution

1. **Percent solution:** This is the most common way of expressing strength of a solution. Percent implies a fixed amount of solute dissolved per hundred parts of final solution.
   It can be further divided as.
   a. *Weight per unit weight (w/w):* In this type weight of both solute and solvent add up to 100, regardless of final volume produced.
   b. *Weight per unit volume (w/v):* In this type both desired weight of solute is dissolved in a solvent to make final volume of 100 ml.
   c. *Volume per unit volume (v/v):* In this type both solute and solvent are liquids. Desired volume of solute is added in a solvent to make final volume of 100 ml.
2. **Part dilutions:** In this method the strength of dilution is expressed as that part of solute dissolved in one part of solvent.
   e.g. RBC dilution is 1:200 that means there is one part of blood in 200 part (by volume) of diluting fluid.
3. **Molar solution:** A molar solution contains the molecular weight of solute in grams per liter of solution
   e.g. The molecular weight of HCl is 36.5 hence one molar solution of acid contains 36.5 gm of HCl in one lit.
4. **Normal solution:** A normal solution contains the gram equivalent weight of a solute per lit. of solution
   e.g. The equivalent weight of sulfuric acid is 49.041. Hence one normal solution of acid contains 49.041gm of acid in one lit.

BUFFER SOLUTIONS

Buffer solutions are generally mixtures of weak acids and their salts of strong base. Such solutions have the ability to accept H^+ and OH^- ions without undergoing any pH change.

Buffer ion plays an important role in restricting pH changes of body fluids.

e.g. Buffers on plasma—

- Bicarbonate—Carbonic acid ($BHCO_3/H_2CO_3$)
- Plasma protein buffer system (B protein/H protein)
- Phosphate buffer system (B_2HPO_4/BH_2PO_4)

Buffers on red blood cells—

- Bicarbonate—Carbonic acid ($BHCO_3/H_2CO_3$)
- Oxyhaemoglobinate— Haemoglobin ($BHbO_2/HHbO_2$)
- Haemoglobinate—Haemoglobin (BHb/HHb)

2 Elementary Knowledge of Organic Chemistry

ORGANIC COMPOUNDS

Organic compounds play an important role in our daily life. To understand various biological processes going on in the human body, basic knowledge of organic chemistry is must. The term organic chemistry is applied to substances, which were derived from animals or plants, i.e living organisms.

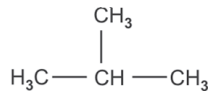
All organic compounds contain carbon as an essential element, combined with hydrogen, oxygen, nitrogen, sulfur, phosphorus and/or halogens.

On the basis of structure, organic compounds are classified into two groups.

i. Acyclic/Open chain/Aliphatic compounds:
ii. Cyclic/Closed chain/Aromatic compounds:

i. Acyclic/Open chain/Aliphatic compounds:

In aliphatic compounds, the molecules are open chained or non-cyclic.
e.g. CH_4,$CH_3 = CH_3$
These compounds may also contain branched chains.
e.g isobutene

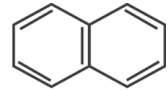


ii. Cyclic / Closed chain / Aromatic compounds:

These compounds contain one or more closed chain of carbon atoms are called cyclic or ring compounds. These compounds possess a pleasant aroma.

e.g.

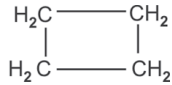
Benzene



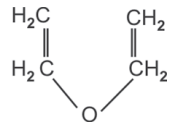
Naphthalene

Aromatic compounds are further sub-divided as:

a. Homocyclic compounds and
b. Heterocyclic compounds
   a. Homocyclic compounds: Homocyclic compounds contain a ring or rings of carbon atoms only.
      e.g. cyclobutane



   b. Heterocyclic compounds: Hetrocyclic compounds contain one or more hetero or non-carbon atoms like oxygen, sulfur, and nitrogen in the rings of the molecules.
      e.g. furan



CLASSIFICATION OF ORGANIC COMPOUNDS

Based Upon Functional Group

Functional group is atom or group of atoms that defines the structure of a particular family of organic compounds. The functional group is responsible for determining the properties of a compound. A particular set of properties is characteristic of a particular kind of structure.

Organic compounds have been classified into a number of classes on the basis of their functional group.

Organic molecules are built up of two parts:

1. One part represents carbon- hydrogen framework and the
2. Other part represents the functional group.

For example, if an organic compound is represented as R–X, R represents the carbon hydrogen frame work and X represents the functional group.

Classes of organic compounds containing C, H, O, N, X, elements

1. *Aryl halides:* These are monohalogen derivatives of alkanes, represented as R–X where X is a halogen (F, Cl, Br, or I) covalently linked to an alkyl group R.
   e.g. $CH_3–CH_2–CH_2–Br$ (Propyl bromide).
2. *Alcohol:* Alcohols are aliphatic organic hydroxy compounds in which hydroxyl group (–OH) is attached to alkyl group.
   e.g. $CH_3–OH$ (methyl alcohol).
3. *Aldehydes:* They are classified as the first oxidation products of primary alcohols. In aldehydes, the carbonyl group is attached to at least one hydrogen atom (–CHO). It is always present at the beginning of carbonyl chain.
   e.g. H–CHO (formaldehyde)
4. *Ketones:* They are the first oxidation products of secondary alcohols. In ketones the carbonyl group is attached to two other carbon atoms i.e, it does not carry hydrogen ($\diagup\!\!\diagdown C = O$). It is always present between the middle of the chain.
   e.g. $CH_3–COO–CH_3$ (dimethyl ketone).
5. *Carboxylic acids:* They are the first oxidation products of aldehydes and ketones and are defined as the acidic organic compounds containing carboxylic group.(–COOH)
   e.g. CH_3—COOH (Acetic acid)
6. *Esters:* Esters are the derivatives of carboxylic acids formed by replacing the hydroxyl group of acid by a carbonyl group. (–OR). These have particular fruit aroma.
   e.g.$CH_3–COO–CH_3$ (Methyl methanoate).
7. *Ethers:* These are alkyl derivatives of alcohols, formed by replacing hydroxyl hydrogen of alcohol.
   e.g. $C_2H_5–O–C_2H_5$ (Diethyl ether)

8. *Amines:* Amines are the derivatives of ammonia formed by replacing one or more hydrogen atoms by corresponding number of alkyl or aryl group radicles.
   e.g. $H–NH_2$ (Ammonia)

Class	General formula	Functional group	Example
1. Alkyl halides	R – X	– X	CH_3I Methyl iodide
2. Alcohol	R – OH	– OH	C_2H_5 OH Ethyl alcohol
3. Aldehydes			HCHO Formaldehyde
4. Ketones			CH_3COH_3C Dimethyl ketone
5. Carboxylic acids			CH_3COOH Acetic acid
6. Esters			$CH_3COO\ C_2\ H_5$ Ethyl acetate
7. Ethers	R – O – R		C_2H_5–O–C H_5 Diethyl ether
8. Amines	$R–NH_2$	$–NH_2$	$C_2\ H_5$ Wh Ethyl amine

Important classes of organic compounds containing only C and H elements are hydrocarbons.The hydrocarbons are the compounds containing only carbon and hydrogen. Hydrocarbons are classified into saturated compounds such as alkanes and unsaturated compounds such as alkenes and alkynes.

1. *Alkane (C_nH_{2n+2}):* The aliphatic saturated hydrocarbons containing carbon carbon single bond are called as alkanes. (≡C–C≡). The general formula is C_nH_{2n+2} where, n is ≥ 1
   e.g. CH_4 (methane) , $C_2\ H_6$ (ethane)

2. *Alkenes (C_nH_{2n}):* The aliphatic unsaturated hydrocarbons containing carbon carbon double bond are called as alkenes. (=C=C=). The general formula is C_nH_{2n} where, n is ≥ 2
   e.g. $C_2\ H_4$ (ethene), $C_2\ H_6$ (propylene)

3. *Alkynes (C_nH_{2n-2}):* The aliphatic unsaturated hydrocarbons containing carbon carbon triple bond are called as alkynes. (–C≡C–). The general formula is C_nH_{2n-2} where, n is ≥ 2
e.g. H–C≡C–H (acetylene), CH_3– C≡C–H (methyl acetylene).

Class	General formula	Functional group	Example
Saturated hydrocarbons (I)Alkanes	C_nH_{2n+2}	$-\overset{|}{\underset{|}{C}}-$	C_2H_6 Ethane
Unsaturated hydrocarbons (ii) Alkenes	C_nH_{2n}	$>C=C<$	$H_2C=CH_2$ Ethene
(iii) Alkynes	C_nH_{2n-2}	$-C\equiv C-$	$H-C\equiv C-H$ Acetylene

3 Elementary Knowledge of Physical Chemistry

OSMOSIS AND DIFFUSION

Osmosis

Osmosis is the spontaneous flow of solvent through a semipermeable membrane from a solution of low concentration to one of higher concentration.

Physiological activities such as absorption from gastrointestinal tract, fluid interchange in various compartments of body (e.g. between plasma and RBC) follow the principle of osmosis.

Osmotic Pressure

The pressure, which must be applied to prevent passage of water molecules through membrane permeable only for water, is called osmotic pressure. The osmotic pressure of a solution depends solely on the number of particles in the solution.

Importance: Concentration of urine is regulated by osmotic pressure. The osmotic effect of plasma proteins causes water to flow from protein free interstitial fluid into the blood vessels.

Types of Solution

i. *Isotonic solution:* Isotonic solutions are a pair of solutions, which produce no flow through semipermeable membrane. These are the solutions with the same osmotic pressure and are termed as iso-osmotics.

For example, living red cells if suspended in 0.85 percent NaCl solution, it neither gain nor loose water.

Thus, an intracellular fluid of red cells is isotonic with 0.85 percent NaCl solution.

ii. *Hypertonic solution:* Hypertonic solution is a solution, which produce flow through semipermeable membrane towards the solution. This results in exosmosis.

For example, living red cells if suspended in 2 percent NaCl solution, which is more concentrated than the concentration of solute inside, the cells will shrink due to exosmosis.

iii. *Hypotonic solution:* Hypotonic solution is a solution, which produce flow through semipermeable membrane towards the cell. This results in endosmosis.

For example, living red cells if suspended in water, which is less concentrated than the concentration of solute inside the cells, it will result in swelling and haemolysis due to endosmosis

DIFFUSION

When a water-soluble substance like sugar or NaCl is added to water it rapidly spreads in the solvent and forms a homogeneous solution. This process is called diffusion.

The uniform spreading over of solute molecules is mainly due to the energy possessed by its molecules.

Diffusion helps in passive transport of substances across the membrane. Substances move in the direction of the physical gradients, which follows the Ficks law. The law is—

$$\frac{ds}{dt} = DA \frac{dc}{dx}$$

where,

$\frac{ds}{dt}$ = rate of movement of solute

D = diffusion constant

A = area of the liquid surface

$\frac{dc}{dx}$ = difference in concentration between any two planes.

Biological Significance of Diffusion

Diffusion is a physiologically important phenomenon of animal life. For example —

1. The mixing of foodstuffs and digestive juices in the gut occurs by diffusion.
2. Absorption of certain foodstuffs in gut occurs by diffusion.
3. Exchange of foodstuffs, oxygen, carbon dioxide between blood and tissue fluid and between tissue fluid and cells occurs by diffusion.
4. Exchange between plasma fluid and RBC occurs by diffusion.
5. Exchange of oxygen and carbon dioxide between lungs and blood is due to diffusion.

COLLOIDS

The term colloid is derived from Greek, Koli = glue and eidos = appearance. Colloids are suspensions of particles that are larger than these true solutions but still smaller to settle out by gravity. They cannot be filtered by ordinary filters, e.g. white of eggs, milk, plasma

A colloidal system consists of two components—

1. Dispersed phase and
2. Dispersion phase

The dispersed phase consists of macromolecular solids like proteins and nucleic acids and liquids like oily fats.

The dispersion phase is the medium in which insoluble materials are dispersed. It may be solid liquid or gas.

Properties of Colloid

1. *Sol and Gel*—a colloid may remain in two states, namely— Sol and Gel. Sol is a liquid and gel is a solid. These two states are reversible.
2. *Brownian movement*—the dispersed particles of colloid constantly move in random zig-zag paths. This haphazard motion of the dispersed particles in dispersion medium is called Brownian movement.

3. *Tyndall phenomenon*—when a beam of light is passed through a colloidal solution, the colloid particles shine due to scattering of light rays. This is called Tyndall phenomenon.
4. Colloid particles carry electric charges, which may be positive or negative.
5. Colloid solution can be precipitated by adding salts, weak acids and weak bases.
6. Colloids exert some osmotic pressure.
7. It shows properties like adsorption and ageing.
8. The colloid particles will not move through semipermeable membrane.

Biological Significance of Colloids

1. Protoplasm exists in colloidal state.
2. Blood plasma, lymph and milk are colloidal emulsions.
3. Blood clotting is a colloidal phenomenon in which sol like plasma is converted into gel like clot.
4. It helps to store water in body.
5. Colloids are dispersed in the form of minute particles. Hence they provide a very large surface area for various reactions to occur, such as enzyme action, adsorption, surface tension, etc.

4 Elementary Knowledge of Analytical Chemistry

BALANCES

Balance is used for determination of mass or weight.

There are many types of balance, for example, platform balance, two-pan analytical balance and single-pan analytical balance. Platform balances are capable of measuring mass to within + 0.05 g. while some analytical balances can measure within 10-4 g.

The precaution in using balance is to minimize vibration. To prevent corrosion of the pans, chemicals should never be placed directly on the metal surface. Always use weighing paper. Care and cleanliness are important for balances. Brush up any spilled chemicals and close the balance-case door when done.

Preparing the Balance for Use

Before weighing anything on the analytical balance you must make sure that it is leveled and zeroed.

To check the leveling on the balance, look at the leveling bubble on the floor of the weighing chamber. If it is not centered, center it by turning the leveling screws on the bottom toward the back of the balance.

Once the balance is leveled, close all the chamber doors and press the control bar on the front of the balance. After a few seconds, a row of zeros will appear. This indicates that the balance is zeroed and ready for use.

These substances must always be weighed using an appropriate weighing container.

1. Place the weighing container on the balance pan and close the doors.

2. Tare the container by briefly pressing the control bar. The readout will read zero with the container sitting on the pan. This allows the mass of the sample to be read directly.
3. Add the substance to be weighed. Be careful not to spill chemicals on the balance.
4. With the sample and its container sitting on the pan, close the chamber doors and read the display to find the mass of sample.

Weighing a Solid Object Directly on the Balance

If the object need to weigh is a solid object, one can weigh it directly on the pan. Be sure the balance is zeroed. Open the chamber doors, carefully place the object on the balance pan, close the doors, and read the mass of object.

Cleaning Up and Shutting Down the Balance

When you are done with the balance, make sure you have properly cleaned up any chemicals that may have spilled on the balance. At the end of the day, the balance can be turned off by lifting up gently on the control bar.

CENTRIFUGE

Centrifuge is used to separate sediments from the fluid by centrifugation. Centrifugation is a process in which a solution is rotated in circles around a central axis. The Centrifugal force and gravitational forces acts together on the solution. This results in separation of sediments and supernatant.

Centrifuges are of three types:

a. Ordinary centrifuge
b. Electric centrifuge, and
c. Ultra centrifuge

a. *Ordinary centrifuge:* These are hands driven centrifuges, which can reach upto 2,000 to 2,500 rotations per minute (rpm). Hand centrifuges can hold only 2 to 4 centrifuge tubes, which are of

15ml. Capacity, and usually made of aluminium. Ordinary Centrifuge produces heat due to friction with air after long run.

The tubes in the centrifuge must be balanced exactly; otherwise the tubes will be broken due to vibration. Gradually increase or decrease the speed of centrifuge. The tubes must be of the same size and weight.

b. *Electric centrifuge:* These are motor driven centrifuge, operated through main electric supply. These can reach upto 3500 rpm and they are usually provided with multi-stage speed regulator to obtain desired speed. The common laboratory centrifuge is used for separation of serum precipitates and sediments of various body fluids, urinary sediments for microscopic examinations, etc.

c. *Ultra centrifuge:* This is a high speed centrifuge. The speed of rotation is more than 60000 rpm. This centrifugal force permits the cell fractionation of sub cellular organelles previously observed only in electron microscope. This in turn permits assay of their enzymatic constituents providing insight into structure-function relationship. Viruses can be isolated in pure form. DNA, RNA and proteins can be analyzed completely.

The ultra centrifuge has drive and speed control. The drive assembly has a water-cooled electric motor connected to the rotor by way of a precision gearbox. The speed rotor may be selected by means of rheostat and monitored by a tachometer. If there is over speed the instrument is automatically shut down. There is an infrared radiometric sensor beneath the rotors, it measures and controls the temperature of the rotors continuously and accurately.

To eliminate heating the rotor is sealed and evaluated by two pumping systems. Friction is completely avoided inside the vacuum.

While spinning, the rotors may be kept at angle of 30^{o} of horizontal.

pH METER

The pH meter is used to measure the pH of a solution.

The pH meter is of two types:

1. Digital pH meter
2. Manual pH meter.

pH meter consists of power pack and two electrodes.

The power pack contains an on/off switch, an indicating meter, a temperature compensation knob, a calibrate knob, and a wire with a plug pin. The on/off switch is used to supply or cut off electric current. The indicating meter shows pH reading. The temperature compensation knob is used to adjust the temperature. The calibrate knob is used to set pH. The plug pin is connected to the main electric line.

The pH meter contains two electrodes— glass electrode and a calomel electrode. In modern pH meters the two electrodes are combined into single unit.

The glass electrode has a hard glass tube. At the base, it has a thin bulb. The bulb contains HCl (0.1 mol/lit.). The bulb is covered by a special membrane of soda glass. It is sensitive to H^+ and it allows H^+ to pass through it. A platinum wire is connected to the HCl through silver – silver chloride electrode. The wire coming out from the glass electrode is connected to the power pack of the pH meter.

The calomel electrode is a reference electrode. It is not sensitive to H^+. It contains a calomel paste. The calomel paste is connected with a platinum wire through mercury. The free end of the calomel electrode has a porous plug. The base of electrode is deposited with KCl crystal. The remaining portion is filled with KCl solution.

The glass electrode in the test solution constitutes a half-cell and the calomel electrode constitutes the other half-cell. The two electrodes complete the circuit.

Principle

If the conductor is immersed into an appropriate electrolyte solution there will be a tendency for its atoms to leave the surface and enter the solution as ions. This is called a half-cell and the conductor is called an electrode. The potential difference between the two half-cell is called the electromotive force. (emf). The emf will change if the flow of electrons is changed.

This is what happens in the measurement of pH. The indicator electrode is dipped in a solution whose hydrogen ion concentration to be measured. H^+ ions are drawn towards the outer surface of the glass membrane. The number of H^+ ions accumulating on the membrane depends on their concentration in the external solution.

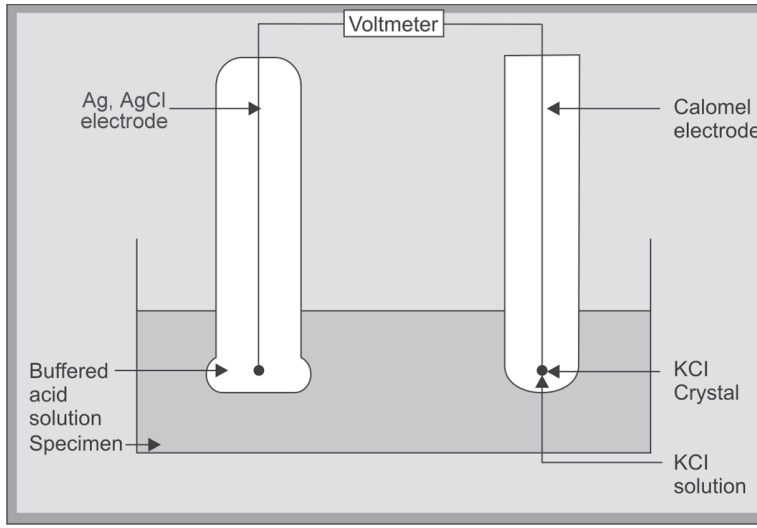


Fig. 4.1: pH meter

The change in the hydrogen ion activity outside the glass bulb changes the potential between the indicator electrode and the calomel electrode. The potential is measured by sensitive voltmeter (Fig. 4.1).

The instrument must be calibrated against buffers of known pH before measuring the pH of unknown specimen.

COLORIMETER

Colorimeter is an instrument used to determine the concentration of biochemical compound by measuring the intensity of colour developed. This method of analysis is called colorimetry.

The colorimeter has a tungsten lamp. The white light from the lamp passes through a slit to a condenser lens. The lens allows parallel beam. The beam falls on a test solution kept in a cuvette. Beyond the cuvette a filter is placed. The filter allows maximum transmission of the colour absorbed. The filter is selected according to the colour of solution. The light then falls on to a photocell. It generates an electric current in direct proportion to the intensity of light falling on it. This small electrical signal is increased in strength by the amplifier. The amplifier signal passes to a galvanometer or digital read out (Fig. 4.2).

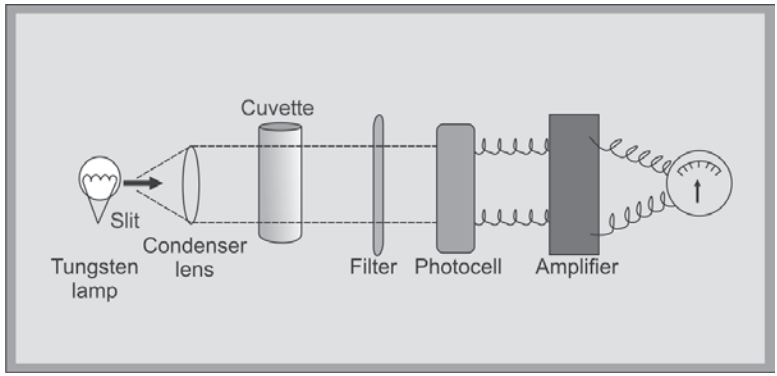


Fig. 4.2: Colorimeter

The instrument contains a wire with plug pin, an on/off switch, a cuvette holder and a zero set knob.

The wire is connected to the main power line. The switch is kept on. Zero set knob is adjusted to set galvanometer zero with the help of blank. The reading in the meter is optical density of the solution.

Principle

The Colorimeter works under the Beers-Lambert law. According to this law when a ray of monochromatic light passes through an absorbing medium, its intensity decreases exponentially as the concentration of the absorbing medium increases.

The filter gives narrow transmission and approximate to monochromatic light. The filter is chosen so that Beers law is obeyed. It is selected according to the colour of the solution.

For example, If a blue solution is under examination then red colour is absorbed and red filter is selected.

The colour of filter should be therefore complementary to the colour of the solution under investigation.

Colour of Solution	*Filter*
Red-Orange	Blue-Blue Green
Blue	Red
Green	Red
Purple	Green
Yellow	Blue
Yellow-Green	Violate

Basic Term Used In Colorimetry

1. *Blank solution:* This is used to set photometer to zero absorbance (A) or 100 percent transmittance (% T). This can be distilled water or the reagent solution if coloured.
2. *Standard solution:* These are solutions of known concentration which range within the limits found in specimen.
3. *Cuvette:* These are small test tube like containers used in colorimeter. It is filled with the solution of which optical density is to be measured and kept in the cuvette holder of the colorimeter. The upper end of the cuvette has an arrow mark. The solution is filled up to this mark. The cuvette is made up of glass/silica/plastic. The sides of the cuvette facing the beam are cut parallel to each other.

Procedure

Set the instrument to zero percent transmission setting with the help of zero setting knob.

1. Pour the blank in a cuvette and set the instrument to 100 percent T with the blank setting knob.
2. Take the absorbance readings of various standard solutions of different concentrations.
3. Each time the cuvette should be rinsed in distilled water and the outer surface of the cuvette is wiped off with soft tissue paper before placing in the cuvette holder.
4. Record the readings in a tabular form – absorbance readings against the sequential concentration of standard used.
5. Plot the calibration curve on linear graph paper for absorbance readings (Y-axis) against various concentration of standard (X-axis). Calibration curve should be at angle of 45°.

Calculation

Results are either calculated by O.D. of test and standard or prepared from calibration curve.

SPECTROPHOTOMETER

Principle

When a beam of incident light of intensity I_o passes through a solution, a part of it is reflected (I_r), a part is absorbed (I_a), and rest is transmitted (I_t).

$$\text{Thus, } I_o = I_r + I_a + I_t$$

In colorimetric methods, I_r is eliminated because the measurement of I_o and I_t is sufficient to determine I_a. For this purpose, the amount of light reflected (I_r) is kept constant by using cells that have identical properties. I_o and I_t are then measured.

The mathematical relationship between the amount of light absorbed and the concentration of substance can be shown by the following two fundamental laws on which spectrophotometry is based.

1. Lambert's Law

This law states that the amount of light absorbed is directly proportional to the length or thickness of solution under analysis.

$$\therefore \quad A = \log_{10} \frac{I_o}{I_t} = a_s b$$

Where, A = absorbancy
a_s = absorbancy index characteristic of the solution.
b = length / thickness of solution

2. Beer's Law

This law states that the amount of light absorbed is directly proportional to the concentration of solute in the solution.

$$\log_{10} \frac{I_o}{I_t} = a_s c$$

The combined Lambert's-Beer's law becomes——

$$\log_{10} \frac{I_o}{I_t} = a_s bc$$

If b is kept constant by applying cuvette or standard cell then,

$$\therefore \quad \log_{10} \frac{I_o}{I_t} = a_s c$$

The absorbancy index a_s is defined as,

$$a_s = \frac{A}{cl}$$

Where,

c = concentration of absorbing material in gm/ lit
l = distance in cm travelled by the light in the solution.

Light absorption in terms of molar concentration of the absorbing material i.e, the molar absorbancy index , a_m

$$a_m = a_s M$$

where,

M = molecular weight of absorbing material.

The Instrument and Procedure

A spectrophotometer has two fundamental parts—a source of radiant light and a monochromator. It consists of a prism that dispenses the radiant energy into a spectrum. A slit is also fixed which selects a narrow portion of the spectrum. The standard cell or cuvette is placed in a light tight unit. The incident light strikes the cuvette and emergent light passes into a measurable electric energy.

The procedure and calculation part of spectrophotometry is same as that of colorimetry.

Applications

This technique may be applied in:

1. Determining the concentration of a compound by measuring the optical density provided the absorancy index i.e, a_s is known.
2. The course of reaction can be determined by measuring the rate of formation or disappearance of a light-absorbing compound.
3. A compound can be identified by determining its absorption spectrum in the visible and UV region of the spectrum.

FLUORIMETER (FIG. 4.3)

Fluorimeter is based on the principle of measuring the intensity of emitted light due to fluorescence. A fluorescent compound is capable of absorbing light of higher energy (shorter wavelength like UV) and emits light of longer wavelength with lower energy.

The components of fluorimeter are:

1. *Source of light:* It is usually in the UV range and emits light of high energy. The use of xenon lamp is common and gives out radiant energy of discrete wavelength.
2. *Primary filter:* This screens the radiant energy coming out of light source. The filter works like the monochromator that allows selected wavelengths of light in the UV region to pass through. This screened light then hits the specimen and excites the organic compound under study.
3. *Specimen carrier:* This carries the specimen. The test compound present in the specimen is capable of excitation that leads to its fluorescence. The primary filter is absorbed by the specimen and used up in the process of fluorescence. The specimen then emits the light of low energy.

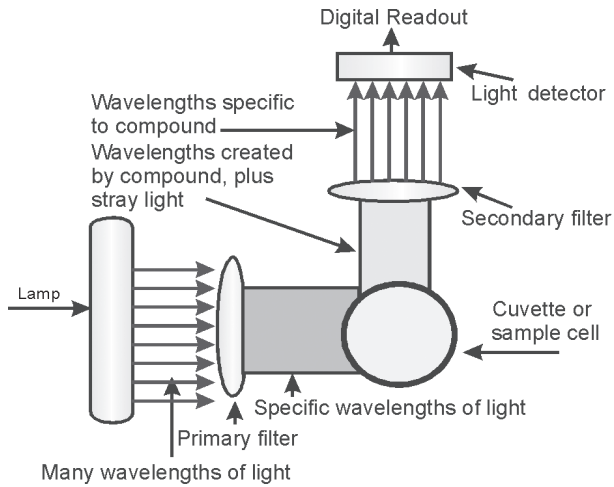


Fig. 4.3: Fluorimeter
(For colour version see Plate 1)

4. *Secondary filter:* This filters the light in the visible range that is emitted by the organic compound present in the specimen. The secondary filter is kept at right angles to the path of exciting light.
5. *Detector:* This measures the amount of light coming through the secondary filter, which is proportional to the concentration of the fluorescing compound. This can either give the reading in the absorbance scale or an arbitrary linear scale reading (0–100)

As like any other photometric analysis, a calibration curve is made with known concentration of the compound under study. The concentration of unknown is then figure out from the photometric readings of the standard.

Applications

1. This method is highly sensitive and a valuable tool in the toxicology laboratory.
2. It is also used in the analysis of various hormones.
3. It is used to determine uranium in salts, determine aluminium in alloys, estimation of boron in steel, etc.

FLAME PHOTOMETRY

Flame photometry is based on the measurement of intensity of the light emitted when a metal is introduced into a flame.

Principle

When a liquid sample containing a metallic salt solution is introduced into a flame, the process involved in flame photometry are –

i. The solvent is vaporized, leaving particles of the solid salt.
ii. The salt is vaporized or converted into gaseous state.
iii. A part or all of the gaseous molecules are progressively disassociated to give free neutral items or radicals. These neutral items are exited by the thermal energy of the flame. The exited atoms, which are unstable, quickly emit photons and return to lower energy state eventually reaching the unexcited state.

When flame photometry is employed as an analytical tool, the wavelength of the radiation coming from a flame tells us what the element is, and the intensity of colour tells us how much of the element is present.

Instrumentation

The components of flame photometer are:

i. *Nebulizer:* This helps to spray the specimen into the burner. It is usually of the scent type; where by a forced string of air passes over a capillary tube that dips into the test solution.

ii. *Flame:* The flame generates heat that volatilizes the element that becomes luminous when it returns to the ground state. The most common gas mixture that provides optimum temperature for the routine determination of sodium and potassium is propane- butane or natural gas. The mixture of fuel, gas and air in right proportions is ignited which provides the flame.

iii. *Monochromators:* These devices increase specificity of the analysis. These are simple filters which screens out all other wavelengths of light except the specific one emitted by the element analyzed, e.g. filters that yield 589 nm for Na and 767 nm for K.

Along with the Monochromator filters, heat filters are placed between the flame and Monochromator filter to stop the passage of heat.

iv. *Photodetectors:* The photodetector system quantifies the emitted light by converting it into an electrical impulse, which is eventually transmitted to the read out galvanometer.

Procedure

Use of an internal standard like lithium, strontium or cesium can minimize the interference and interaction of other elements present in serum and excited simultaneously. The element used as internal standard is not normally present in serum.

First of all dilute the specimen and standard (1:200). If the internal standard is to be used, the specimen and the standard are diluted with the internal standard solution.

Turn on the electrical connections of the flame photometer. Allow it to stabilize for few minutes. Turn on the air compressor and open the fuel valve.

Ignite the flame by pressing ignition button.

Put the de-ionized water under the nebulizer inlet tube. Zero the panel reading. Set the internal standard (Lithium) indicator at the required level.

Replace the water with standard solution and set the levels for sodium and potassium.

Put into the diluted specimen and take the readings. Most instruments are directly read out and do not require a calibration curve.

Applications

1. Flame photometry is a simple, rapid method for the routine determination of elements that can be easily excited. It provides high sensitivity and high reliability for the determination of elements in the first two columns of the periodic table. Among these elements are sodium, potassium, lithium, calcium, magnesium, strontium and barium. The measurement of these elements is very useful in determining certain transition elements, such as copper, manganese and iron.
2. The main use of flame photometer in clinical biochemistry is to determine level of sodium and potassium from serum.
3. Ion selective electrode is used to determine chloride ion. It is seen more automated instruments in conjunction with the determination of sodium and potassium. It is based on potentiometry. (pH meter-principle)

URINOMETER

It is a hydrometer for measuring the specific gravity of urine. It uses a glass float weighted with mercury and a stem with calibrations to measure the buoyancy. Specific gravity of a solution is the ratio of that solution relative to the weight of an identical volume of water. The normal range for specific gravity of urine is 1.010 to 1.030. The specific gravity of urine gives an estimate of the concentration of urinary solids. Long's coefficient (2.66) times the thousands digits (last two digits) of the specific gravity gives the estimate of total solids in grams per liter. A specific gravity of 1.018 gives an estimate of 47.88 grams/liter for urinary solids.

Fig. 4.4
Urinometer
(For colour version see Plate 1)

Fill a urinometer cylinder (Fig. 4.4) to about 1 inch from the top with the collected urine. Holding the urinometer float by the stem, slowly insert it into the cylinder. Do not wet the stem above the water line or an inaccurate reading will result. Give the float a slight

swirl and read the specific gravity from the graduated marks on the stem as it comes to rest. Do not accept a reading if the float is against the side of the cylinder. Read the specific gravity to the nearest 0.001 according to the scale. Read at the bottom of the meniscus. Also measure the temperature. The urinometer is calibrated at 15°C. For each 3°C deviation above 15°C add 0.001 to the reading and subtract 0.001 from the reading for each 3°C below 15°C. Rinse and carefully dry the urinometer and thermometer.

The main drawback of using urinometer is—it requires large volume of urine,it is time consuming and the specific gravity of a solution is dependent on temperature.

CHROMATOGRAPHY

Chromatography is a biochemical technique in which the components of mixture are separated based on their differential migration rates. In this method various biomolecules such as amino acids, carbohydrates etc. can be separated and identified.

Chromatography involves two main phases/media, namely stationary phase and a mobile phase. The stationary phase is a supporting medium such as filter paper or silica gel. The mixture to be separated (solute) is adsorbed on stationary phase. The mobile phase is called the solvent system. It consists of a mixture of two solvents. One solvent is usually water, which has a stronger affinity to stationary supporting system. The other solvent has lesser affinity to the stationary phase and it carries the components of the mixture (solute) across the stationary phase.

Principle

Chromatography is based on the following two factors:

1. Different rates of migration of the components of a mixture on a solvent system.
2. Different rates of adsorption of the components on the stationary phase.

Types of Chromatography

i. Paper Chromatography
ii. Thin layer Chromatography
iii. Column Chromatography
iv. Gas Chromatography

Paper Chromatography

Paper Chromatography involves the use of filter paper as the supporting medium (Fig. 4.5) .

A strip of rectangular filter paper is suspended vertically. The lower end is allowed to dip into the solvent .The mixture to be separated is spotted at the lower end of the paper above the surface level of the solvent.

The solvent moves up the filter paper. The components are also carried along with the solvent. The components migrate at different rates depending upon their solubility in the solvent to certain distances on the filter paper. Afterwards they stop migrating. But solvent rises up leaving behind the components of the mixture.

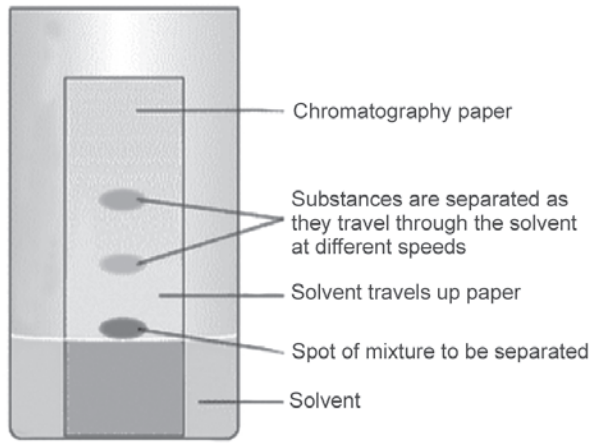


Fig. 4.5: Paper chromatography
(For colour version see Plate 2)

The run is stopped as the solvent reaches near the upper edge of filter paper. The strip of filter paper is removed and the position of solvent called solvent front is marked on the filter paper.
The filter paper is dried and stained with ninhydrin (in case of proteins and amino acids). The positions of different spots are observed.

The individual components are identified by their Rf (Resolution front) values. Rf value is the migration rate. It is defined as the ratio of distance of the spot from the spotted line to the distance of the solvent from the spotted line.

$$\text{Rf value} = \frac{\text{Distance of the spot from the spotted line}}{\text{Distance of the solvent from the spotted line}}$$

Rf values are constant for different amino acids when chormatographed under the same experimental condition.

Thin Layer Chromatography (TLC)

Thin layer Chromatography is a modification of paper Chromatography. In this technique silica gel or alumina is used instead

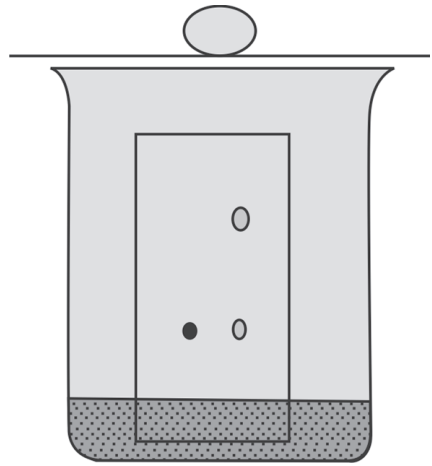


Fig. 4.6: Thin layer chromatography

of filter paper. It is spread on a glass plate as a thin layer and then dipped in a tray and kept in a jar with lid (Fig. 4.6).

Column Chromatography

In column chromatography a long glass tube (20 cm × 1 cm) is used. The glass tube is packed with a insoluble material such as calcium carbonate.

The stationary phase is solid and the mobile phase is liquid.

The mixture to be separated is applied on the top of the column. The solvent to be poured on the top of the column. The components of the mixture are move down along with the solvent.

The effluent coming out from the column is collected into a series of test tubes. Each test tube contains the separated components of the mixture (Fig. 4.7).

Gas Chromatography

In gas chromatography inert gas is used as the mobile phase. Gases like hydrogen, helium, argon, neon are used. The stationary phase may be solid or liquid. It includes solids like alumina or liquids like silicon oil or polyethylene glycol.

The stationary phase is filled in a long coiled metal or glass tube.

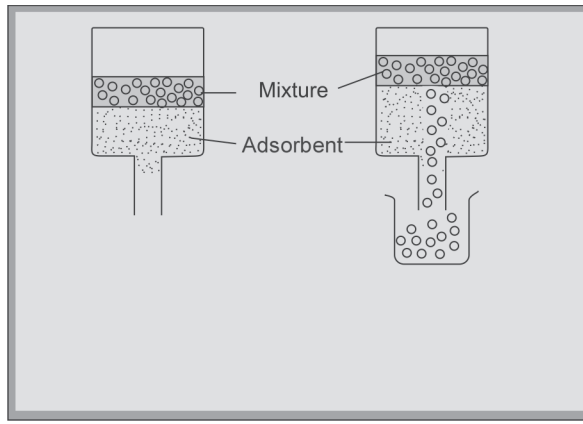


Fig. 4.7: Column chromatography

It is called the column. The mixture to be separated is injected into the tube. This is followed by injection of mobile phase. The mixture is volatized by high temperature (175 to 200°C) and is swept through stationary phase by the gas. The individual components are adsorbed and retained by the stationary phase at different rates depending upon their affinity to the stationary phase. Consequently the components, which have lesser affinity to the stationary phase, move through the column at a quicker rate and emerge out of the column earlier than those who have stronger affinity.

The different components emerge out at different intervals and are recorded on an automatic recorder and analyzed.

Gas chromatography is used for the separation of volatile substances. In clinical biochemistry this is used for the separation of plasma lipids, blood alcohol, urinary steroids etc.

ELECTROPHORESIS

Electrophoresis is a method of spreading a mixture of organic compounds. It is used to separate proteins like haemoglobin, serum protein, lipoprotein, and isoenzyme. It is most useful in the diagnosis of haemoglobinopathies.

Principle

Migration of ions in an electrical field at definite pH is called electrophoresis. It is based on a principle that proteins migrate in an electric field except at the pH of their isoelectric point.

Instrumentation (Fig. 4.8)

The electrophoresis apparatus consists of two electrode chambers filled with a buffer solution. The mixture to be separated is spotted at the center of a strip of Whatman filter paper. The center of filter paper is supported over a glass rod. The ends of the paper dipped into the buffer solution. The apparatus is covered with a lid. When electric current is passed, the charged particles present in the mixture migrates towards the electrodes. The anions move towards anode and cations move towards cathode (Fig. 4.8).

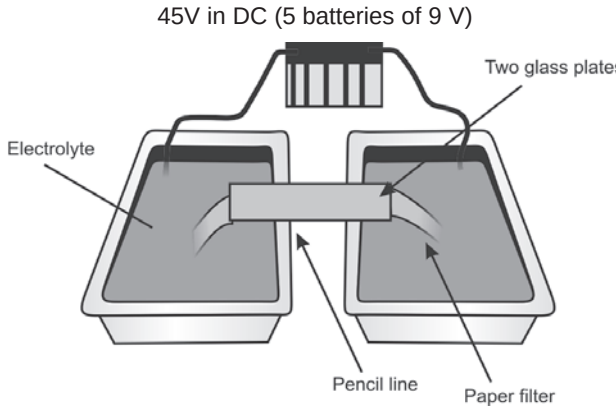


Fig. 4.8: Apparatus for paper electrophoresis
(For colour version see Plate 2)

The charged particles move at different rates and they are fixed on the filter paper at different zones. The filter paper is stained and components of mixture are identified.

Applications

Electrophoresis is mainly used to separate proteins.

The protein has both amino group as well as carboxyl groups that can potentially carry positive or negative charges depending on whether the pH of the medium is acidic or alkaline. At the isoelectric point, when protein bears equal amounts of +ve or –ve charges, it behaves as neutral. If the pH is above isoelectric point, the protein carries a negative charge and will move towards positive pole. (cathode)

During electrophoresis, the pH of the buffer is kept at 8.4, as the mixture of serum proteins is placed on a spot of support medium and when a current is passed, in different proteins (albumin, globulins, beta and gamma, alpha proteins etc.) migrates at different rates. This is because each has a different isoelectric point (4.7 for albumin to 7.3 for globulin). The location of protein spot is determined by staining with a protein staining dye.

Electrophoresis of haemoglobin is done with the goal of identifying abnormal haemoglobin. Migration rate of abnormal haemoglobin varies from the normal HbA. Thus they can be identified.

DENSITOMETER

A densitometer is a device that measures the degree of darkness (the optical density) of a photographic or semitransparent material or reflecting surface. The densitometer is basically a light source aimed at a photoelectric cell, which determines the density of the sample from differences in the readings. Modern densitometers have the same components, but also have electronic integrated circuitry for better reading.

There are two types:

- Transmission densitometers that measure transparent materials
- Reflection densitometers that measure light reflected from a surface.

Some modern types of German manufacture are capable of both types of measurements selectable by a switch. They are used in analog photography to measure densities of negatives with the switch in the "T" (Transmission) position and the saturation of a resulting print in the "R" position. Such measurements enable the photographer to choose the right photo paper and the correct exposure, obviating experiments with test strips. Once the papers and darkroom have been calibrated, the first print from a previously measured negative is a success at once.

Densitometers are used for measuring color saturation by print professionals, and calibrating printing equipment. They are also used for making adjustments so outputs are consistent with the colors desired in the finished products.

Part II
Clinical Biochemistry

5 Aim and Scope of Biochemistry

The term 'biochemistry' was first introduced by a German chemist Carl Neuberg in 1903.

Biochemistry may be defined as a science concerned with the chemical nature and chemical behavior of the living matter. It takes into account the studies related to the nature of the chemical constituents of living matter, their transformations in biological systems and the energy changes associated with these transformations.

The knowledge of biochemistry is growing speedily; newer disciplines are emerging from the parent biochemistry. Some of the disciplines are enzymology (science of study of enzymes), endocrinology (Science dealing with endocrine secretions or the hormones), Clinical biochemistry, and Molecular biochemistry etc. Along with these branches certain link specialties have also come up such as Agricultural biochemistry, Pharmacological Biochemistry etc.

Modern biochemistry is relatively a new branch and much of the work has been conducted recently. The biochemistry has developed with correlation of physical events in biological systems with the help of chemical processes. The aim of biochemistry is to find out the physical relations along biochemical substances and of these substances to the environments. Thus, principal objective of biochemistry is to fill the wide gap between the highly intricate functions of the living cell and the various properties of its individual chemical constituents. A Biochemist therefore has to perform an important arduous task carrying the research work with utmost sincerity, patience and honesty.

The American society of biological chemist has defined biochemist as – 'A biochemist is an investigator who utilizes chemical, physical or biological techniques to study chemical nature and behavior of living matter.

SCOPE OF BIOCHEMISTRY

Biochemistry has more advanced since mid 20th century with the development of new techniques such as chromatography, X-ray diffraction, NMR spectroscopy, radio isotopic labeling, electron microscopy and molecular dynamics simulations. Clinical biochemistry is an analytical and interpretative science. The analytical part involves the determination of the level of chemical components in body fluids and tissues. The interpretative part examines these results and uses them in the diagnosis of disease, the screening for susceptibility to specific diseases, and monitoring of progress of treatment.

Advances in biochemistry have found large-scale applications in various areas industry, agriculture, medicine and pharmacy.

1. *Genetic engineering:* By applying biochemical techniques, improved strains of domestic animals and cultivated plants are produced through genetic engineering, nitrogen fixing genes of bacteria are transferred to higher plants (wheat and maize).

2. *Feed proteins:* New strains of microorganisms are being employed in the production of low-cost feed protein and essential amino acids for domestic animals.

3. *Biological control:* To protect cultivated plants from the pests, biological preparations of superior quality that are not harmful to human or animals are currently being manufactured.

4. *Control of pollution:* Biological methods for the disposal of industrial and domestic wastes and for the cleaning up oil spills, common contaminants of the seas (using specially cultivated bacterial mutants) have proved to be of substantial value.

5. *Industrial development:* Biochemical processes are widely used in the food industry (in the preparation of bread, cheese, wine etc.) and in the leather industry. Detergents with enzyme additions are also commonly available.

6. *Medical field:* Biochemical methods are increasing by gaining acceptance in pharmaceutical practice. Enzymes are used in technology for the synthesis of drugs such as steroid hormones.

The clinical biochemistry may aid in:

a. Discovering occult disease.
b. Preventing irreparable damage.
c. Early diagnosis after onset of signs or symptoms.
d. Differential diagnosis of various possible diseases.
e. Determining the stage of the disease.
f. Estimating the activity of the disease.
g. Detecting the recurrence of the disease.
h. Monitoring the effect of therapy.
i. Genetic consulting in familial problems.
j. Medico-legal problems, such as paternity suits.

The application of bacterial techniques has enabled scientists to develop convenient and economical methods for the commercial synthesis of pharmaceuticals such as insulin, vitamins, antibiotics, antibodies, amino acids, nucleotides, nucleosides etc.

6 Structure of Cell

The structure and functions of each cell-organelles are as follows:

Plasma Membrane (Plasmolemma)

A typical animal cell is bounded by a thin membrane known as Plasma membrane or cell membrane, which is of 75 °A thickness. Plasma membrane is made up of thick layers.

The outer layer is made up of proteins and polysaccharides; the middle layer consists of phospholipids and cholesterol level, while the inner layer contains only proteins.

Nature of lipo-proteins of cell membrane decides the change in membrane and the movement of molecules across the membrane. It is semipermeable and encloses the cytoplasm in which various and very important organelles are present.

Nucleus and Nucleolus

A dense spherical structure called *nucleus* is present in the center of the cell. It has a double-layered nuclear membrane called nuclear envelope. The nucleolus is composed of two important nucleic acids, the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). RNA is in granular form and DNA is in chromosome form.

Within the nucleus a single or more spherical bodies called nucleoli are present. The nucleolus is the aggregation of portions of chromosomes, which are responsible for the secretion of the ribosomes, the sub–units. Inside the nuclear membrane the living matter is called nucleoplasm. The double layered nuclear membrane

is porous and provides a continuity between cytoplasm and the contents of the nucleus (chromosomes).

The outer nuclear membrane bears ribosomes, which may continue with the membrane of endoplasmic reticulum.

Mitochondria

Within the cytoplasm, there are numerous double-layered elongated bodies called mitochondria. It is composed largely of proteins and lipids. These are responsible for transformation of chemical energy into biological energy in the form of ATP compounds. All enzymes involved in Krebs' cycle are present in mitochondria. It is also responsible for transmission of heredity characters. Each mitochondrion is composed of tubular or paired lamellae called cristae. It is the site for carbohydrate and lipid (fat) metabolism, i.e. respiration.

Golgi Apparatus

The Golgi apparatus (body) is compact and consists of parallel membrane plates and tubules. It is the site for enzyme secretion. It participates in the formation of lysosomes.

Endoplasmic Reticulum and Ribosomes

Within the cytoplasm of the cell is an extensive network of membrane arranged in plates and tubules, collectively known as the endoplasmic reticulum. It is the site for biochemical synthesis and intracellular transport of molecules.

The endoplasmic reticulum present is attached to the nuclear membrane and the plasma membrane. On the membrane of endoplasmic reticulum, there are small round bodies present called ribosomes. It is the site for protein synthesis. The endoplasmic reticulum with the ribosomes is called the rough endoplasmic reticulum, while the other devoid of ribosomes it is called the smooth endoplasmic reticulum.

Lysosomes

The lysosomes are small vesicular structures containing homogeneous fluid. It is composed of a single layer and contains digestive enzymes for intracellular digestion.

Microtubules

These are tubular structures composed of globular protein and held in intracellular transport.

Microfilaments

These are protein filaments in the cytoplasm meant for contraction and mobility of the cell.

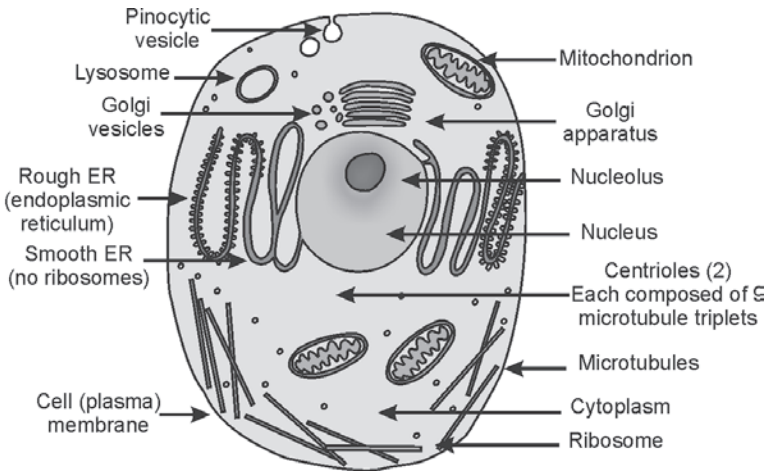


Fig. 6.1: The animal cell
(For colour version see Plate 3)

The animal cell (Fig. 6.1) is responsible to perform functions like motility, digestion, metabolism, growth, reproduction and irritability.

7 Carbohydrates

Carbohydrates constitute the greatest proportion of organic material on earth. They are the main components of the food. Carbohydrates are the important components of our clothing, housing furniture, etc.

Carbohydrates are defined as polyhydroxy aldehydes and ketones and their derivatives.

CLASSIFICATION

Carbohydrates are classified as:

1. *Monosaccharides:* These are the simplest members of Carbohydrate, which are represented by the general formula — $C_nH_{2n}O_n$. Monosaccharides are grouped according to the number of carbon atoms present in a sugar molecule such as trioses (3), tetroses (4), pentoses (5), and hexoses (6). Each of these can be further named as aldoses or ketose depending on the presence of aldehyde or ketone group respectively
   For example: Glucose

2. *Oligosaccharide:* These are condensation products of two to ten simple sugars or monosaccharide. They are represented by general formula $C_n(H_2O)_{n-1}$. Oligosaccharides are grouped according to number of monosaccharide units present in a molecule.
   For example: Maltose, Lactose and Sucrose are di-saccharides and Raffinose is Tri-saccharide.

3. *Polysaccharide:* These are the majority of carbohydrates found in natural sources. These are high molecular weight polymers of monosaccharides represented by general formula $(C_6H_{10}O_5)n$. They are classified as homopolysaccharide and heteropolysaccharide

depending on the presence of either the same monosaccharide or more than one simple sugar.

For example: Cellulose, Glucogen are homopolysaccharides and Glucuronic acid is a heteropolysaccharide.

DETECTIVE TESTS FOR CARBOHYDRATE

1. *Dehydration:* Carbohydrates on dehydration give furfural or its derivatives. Concentrated sulfuric acid is used as dehydrating agent.

   For example: Molish Test – It is general test for carbohydrate identification. Furfural or its derivatives formed during dehydration react with α-naphthol to give violet colour.

$$\text{Ribose} \xrightarrow{\text{conc. } H_2SO_4} \text{Furfural}$$

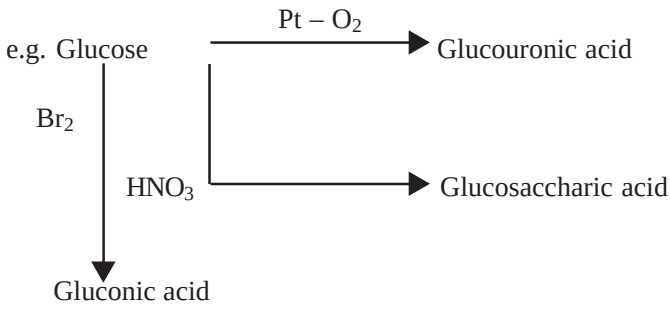
2. *Reducing properties:* Carbohydrates having free cabonyl group acts as reducing agent. It reduces certain metal ions like copper, mercury etc. based on this property following tests detect reducing sugars.
   i. *Benedict's test:* Carbohydrates is heated with alkaline copper sulphate, Cu^{++} ions get reduced and give red precipitate of Cu_2O.
   ii. *Fehling's test:* Reducing sugar reduces Cu^{++} present in Fehlings solution so as to give red precipitates of Cu_2O.
   iii. *Formation of osazone:* When reducing sugar is heated with phenylhydrazin, yellow crystalline compounds called osazones are formed. Definite crystalline forms of osazone are obtained from different reducing sugars.

   For example: Glucose, Phenylhydrazine react with two molecules of phenylhydrazine to give glucosazone, aniline and ammonia.

3. *Reduction:* The carbonyl group of sugar can be reduced by variety of reagents such as H_2 and Pt to an alcohol such carbohydrate derivatives are called alditols.

$$\text{e.g. Glucose} \xrightarrow{H_2 - Pt} \text{Sorbitol}$$

4. *Oxidation:* Sugar on oxidation gives acid. The oxidation product depends upon oxidizing agent used in the reaction.



5. *Mucic acid test:* This test is used for identification of galactose and lactose. Galactose/lactose on oxidation in presence of concentrated nitric acid gives galactosaccharic acid (mucic acid). The mucic acid is insoluble and gets crystallized. The crystals are colourless and broken glass type.

6. *Iodine test:* Iodine reacts with different sugars to form different colours.

Example:

Types of polysaccharide	*Colour with iodine*
Starch	Blue
Dextrine	Brown
Glycogen	Pink
Amylase	Deep Blue
Amylopectin	Purple

METABOLISM

Glucose is the major energy source in most life forms; a number of catabolic pathways converge on glucose. For instance, polysaccharides are broken down into their monomers (glycogen phosphorylase removes glucose residues from glycogen). Disaccharides like lactose or sucrose are cleaved into their two component monosaccharides. Glucose is mainly metabolized by a very important and ancient ten-step pathway called glycolysis, the net result of which is to break down one molecule of glucose into two molecules of pyruvate; this also produces a net two molecules of ATP, the energy currency of cells, along with two reducing equivalents in the form of converting

NAD^+ to NADH. This does not require oxygen; if no oxygen is available (or the cell cannot use oxygen), the NAD is restored by converting the pyruvate to lactate (e.g. in humans) or to ethanol plus carbon dioxide (e.g. in yeast). Other monosaccharides like galactose and fructose can be converted into intermediates of the glycolytic pathway. In aerobic cells with sufficient oxygen, like most human cells, the pyruvate is further metabolized. It is irreversibly converted to acetyl-Co-A , giving off one carbon atom as the waste product carbon dioxide, generating another reducing equivalent as NADH. The two molecules acetyl-Co-A (from one molecule of glucose) then enter the citric acid cycle, producing two more molecules of ATP, six more NADH molecules and two reduced (ubi) quinones (via $FADH_2$ as enzyme-bound cofactor), and releasing the remaining carbon atoms as carbon dioxide. The produced NADH and quinol molecules then feed into the enzyme complexes of the respiratory chain, an electron transport system transferring the electrons ultimately to oxygen and conserving the released energy in the form of a proton gradient over a membrane (inner mitochondrial membrane in eukaryotes). Thereby, oxygen is reduced to water and the original electron acceptors NAD^+ and quinone are regenerated. This is why humans breathe in oxygen and breathe out carbon dioxide. The energy released from transferring the electrons from high-energy states in NADH and quinol is conserved first as proton gradient and converted to ATP via ATP synthase. This generates an additional 28 molecules of ATP (24 from the 8 NADH + 4 from the 2 quinols), totaling to 32 molecules of ATP conserved per degraded glucose (two from glycolysis + two from the citrate cycle). It is clear that using oxygen to completely oxidize glucose provides an organism with far more energy than any oxygen-independent metabolic feature, and this is thought to be the reason why complex life appeared only after Earth's atmosphere accumulated large amounts of oxygen.

In vertebrates, vigorously contracting skeletal muscles (during weightlifting or sprinting, for example) do not receive enough oxygen to meet the energy demand, and so they shift to anaerobic metabolism, converting glucose to lactate (lactic acid). The liver regenerates the glucose, using a process called gluconeogenesis. This process is not quite the opposite of glycolysis, and actually requires three times the amount of energy gained from glycolysis (six molecules of ATP are

used, compared to the two gained in glycolysis). Analogous to the above reactions, the glucose produced can then undergo glycolysis in tissues that need energy, be stored as glycogen (or starch in plants), or be converted to other monosaccharides or joined into di- or oligosaccharides.

DISEASES RELATED TO CARBOHYDRATE METABOLISM

Various diseases have been reported due to abnormal carbohydrate metabolism. Though carbohydrates are main source of energy, they are also required for several other functions–

1. Deficiency of few enzymes like uridine-diphosphate-4-epimerase or galactose-1-phosphate uridyl transferase or galactokinase in the body may not perform the conversion of galactose to glucose which results in GALACTOSEMIAS. This is characterized by abnormal renal tubular failure and liver diseases. Galactitol formation and its accumulation in lens leads to cataract.
2. *Diabetes:* When body does not produce insulin, it results in high glucose level of the blood and results in diabetes mellitus. This leads to saturation of glucose in blood. The glucose then passes to urine. This condition is known as glucosuria.
3. *Glycogen storage:* Progressive cirrhosis of liver cells produce a condition in which glycogen does not get converted to glucose and hence, glycogen in blood goes on increasing.
4. *Fructose intolerance:* Fructose is a common sugar present in fruits. Its defective metabolism results in high concentration of fructose in blood. This is called as fructose intolerance.

BIOCHEMICAL IMPORTANCE OF CARBOHYDRATE

1. Carbohydrates are important constituents of the cell structure in the form of glycolipid, glycoproteins, heparin, cellulose, starch and glycogen.
2. Carbohydrates serve as an important source and store of energy.
3. Carbohydrates play important role in metabolism of amino acids and fatty acids.
4. Lactose promotes the growth of desirable bacteria in the small intestine. It also increases calcium absorption.

5. They protect friction surfaces such as blood vessels, trachea, etc. against mechanical damage.
6. It plays an important role in maintaining osmotic and ionic regulation of body.
7. It works as an intracellular cementing material.
8. It spares protein.
9. Heparin is a carbohydrate, which works as an anticoagulant in body.

8 Proteins

Proteins are one of the chief constituents of all living organisms. Proteins are defined as high molecular weight mixed polymers of α amino acids joined together with peptide linkage (CO-NH).

The total numbers of amino acids, the sequence in which amino acids are arranged, and the over all three-dimensional structure of the molecule is characteristic of each protein and is responsible for its biological activity.

CLASSIFICATION

Proteins can be classified on the basis of their solubility and composition as follows:

1. *Simple Protein:* These Proteins on hydrolysis yield α amino acids and their derivatives. They are subdivided on the basis of solubility into following groups—
   a. Albumins: These are water-soluble proteins, which coagulates on heating, e.g. egg albumin, serum albumin.
   b. Globulin: These proteins are insoluble in water and soluble in dilute salt solution.
   c. Glutelins: These proteins are insoluble in water and soluble in dilute acids and alkalies. These are mostly found in plants, e.g. Glutenin(wheat) and Oryzenin (rice)
   d. Prolamines: These proteins are insoluble in water and soluble in 70 percent alcohol, e.g. Zein(corn) and Gliadin (wheat)
   e. Scleroproteins: These proteins have structure and protective in function. These are insoluble in all solvents, e.g. keratin (horn,nail,hoof and feathers), collagen(bone and skin)

2. *Conjugated proteins:* Conjugated proteins are those, which on hydrolysis yield a non-protein substance called as prosthetic group. These are subdivided on the basis of prosthetic group attached to proteins.
   a. Nucleoproteins: These proteins occur in combination with nucleic acids, e.g. nucleoproteimines and nucleohistones
   b. Phosphoproteins: These proteins contain phosphorus radical as a prosthetic group, e.g. casein (milk) and vitellin (egg yolk)
   c. Glycoproteins: These contain carbohydrate as a prosthetic group, e.g. mucin (saliva) and ovomucoid (egg white)
   d. Porphyrinoproteins: These are proteins which have porphyrin (with a metal ion) as their prosthetic group, e.g. haemoglobin (Fe)
   e. Lipoproteins: Lipoproteins are combination of proteins with lipids such as fatty acids, cholesterol, etc., e.g. α and β lipoproteins of blood.
   f. Flavoproteins: These are the proteins which have riboflavin as their prosthetic group.
   g. Metalloproteins: These are the proteins which have metal ions as their prosthetic group, e.g. ceruloplasmin (Cu).

3. *Derived proteins:* These are the proteins which arise as a result of partial hydrolysis of proteins or due to removal of prosthetic group or due to other changes in the molecule.
   These proteins have been subclassified as—
   a. Primary derived proteins: These are denatured forms of various proteins formed by action of dilute acids/ezymes/or water, e.g. Meta proteins are formed by the action of slightly stronger acids and alkalies, e.g. Myosan.
      Coagulated proteins are formed by the action of heat, X- rays, UV-rays and alcohol, (e.g. coagulated albumin)
   b. Secondary derived proteins: These proteins are formed due to the hydrolytic cleavage of peptide bonds in proteins, e.g. peptones, peptides.

DETECTIVE TESTS FOR PROTEINS

1. *Heat test:* When a protein solution is heated in a boiling water bath, the proteins get coagulated and loose their biological activity, e.g. boiling of eggs

2. *Test with trichloroacetic acid (TCA):* TCA denatures the protein. It is normally used to precipitate proteins from their solution.
3. *Biuret test:* Biuret reagent consists of copper sulphate in an alkaline medium. When proteins are treated with Biuret reagent, it shows violet colour.
4. *Hydrolysis test:* Protein on hydrolysis gives free amino acids. Hydrolysis can be carried out by acids like HCl, H_2SO_4 etc. or alkalies like NaOH, KOH etc.
5. *Xanthoprotic test:* Nitration of aromatic amino acids of proteins gives yellow colour. Conc Nitric acid can be used for nitration.
6. *Millon's test:* Phenolic group of tyrosine of proteins react with mercuric sulphate in the presence of sodium nitrite and sulphuric acid to give red colour.
7. *Precipitation test:* Proteins are precipitated by using different agents, e.g. salts like sodium chloride, ammonium sulphate organic solvents like acetone, alcohol, heavy metals like Cu, mercury salts acids like TCA, HCl, CH_3COOH.

METABOLISM OF PROTEIN

Ingested proteins are usually broken up into single amino acids or dipeptides in the small intestine, and then absorbed. They can then be joined together to make new proteins. Intermediate products of glycolysis, the citric acid cycle, and the pentose phosphate pathway can be used to make all twenty amino acids.

The pentose phosphate pathway (also called Phosphogluconate Pathway, or Hexose Monophosphate Shunt [HMP shunt]) is a process that serves to generate NADPH and the synthesis of pentose (5-carbon) sugars. There are two distinct phases in the pathway. The first is the oxidative phase, in which NADPH is generated, and the second is the non-oxidative synthesis of 5 carbon sugars. The pathway is one of the three main ways the body creates reducing molecules to prevent oxidative stress, accounting for approximately 10 percent of NADPH production in humans. It reduces the coenzyme glutathione which converts reactive H_2O_2 into H_2O. If absent, the H_2O_2 would be converted to hydroxyl free radicals which can attack the cell.

Most bacteria and plants possess all the necessary enzymes to synthesize them. Humans and other mammals, however, can only synthesize half of them. They cannot synthesize isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These are the essential amino acids, since it is essential to ingest them. Mammals do possess the enzymes to synthesize alanine, asparagine, asparate, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine, the nonessential amino acids. While they can synthesize arginine, and histidine, they cannot produce it in sufficient amounts for young, growing animals, and so these are often considered essential amino acids.

If the amino group is removed from an amino acid, it leaves behind a carbon skeleton called an α-keto acid. Enzymes called transaminases can easily transfer the amino group from one amino acid (making it an α-keto acid) to another α-keto acid (making it an amino acid). This is important in the biosynthesis of amino acids, as for many of the pathways, intermediates from other biochemical pathways are converted to the α-keto acid skeleton, and then an amino group is added, often via transamination. The amino acids may then be linked together to make a protein.

A similar process is used to break down proteins. It is first hydrolyzed into its component amino acids. The conversion of an amino acid to α-keto acid with the liberation of ammonia is called deamination. Free ammonia (NH_3, existing as the ammonium ion (NH_4^+) in blood) is toxic to life forms. A suitable method for excreting it must therefore exist. Different strategies have evolved in different animals, depending on the animal's needs. Unicellular organisms, of course, simply release the ammonia into the environment. Similarly, bony fish can release the ammonia into the water where it is quickly diluted. In general, mammals convert the ammonia into urea, via the urea cycle.

DISEASES RELATED TO PROTEIN METABOLISM

Protein deficiency particularly in early childhood is regarded as the disease. The qualitative and quantitative deficiency of proteins in children's result in the disease Kwashiorkor. It appears most commonly in children between the ages of 1 to 4 years. The protein and other

nutritional deficiencies especially in infants below one year is called marasmus. In adults, protein deficiency disease is very rare as proteins are no longer required for growth. But long continue deprivation of proteins may result in nutritional oedema.

Phenylketonuria—This is generic disorder related to phenylalanine metabolism. Phenylalanine is precursor for the biosynthesis of tyrosine. In this disorder, an inherited deficiency of phenylalanine hydroxylase is seen. This leads to accumulation of phenylalanine and is excreated as phenylpyruvate. This condition is known as phenylketonuria. Due to deficiency of tyrosine, mental retardation is seen in young infants.

Alkaptonuria: It is a rare inborn error of metabolism caused by a lack of enzyme homogentisate oxidase, resulting in the failure to oxidise homogentisate formed during phenlyalanine catabolism. Homogentisate is excreated in urine.

BIOCHEMICAL IMPORTANCE OF PROTEINS

1. Protein is one of the important components of diet. It is required to maintain growth and healthy function of the body.
2. Proteins are structural component of protoplasm, cells and tissues.
3. Enzymes and few hormones are proteinous in nature.
4. Antibodies, haemoglobin are also protein. Protein play, important role in cell-mediated immunity mechanism.
5. Blood cloting is formed by protein-fibrogen.
6. Protein may serve as mechanical support to biological structures, e.g. keratin, collagen
7. The nerve impulses are transmitted through synapse, with the help of receptor proteins.
8. Many hormones are protein, e.g. insulin.

9 Lipids

Lipids are heterogeneous group of oily/greasy organic compounds, which are relatively insoluble in water but soluble in organic solvents. It involves diverse group of compounds.

CLASSIFICATION

1. Simple Lipids
2. Compound Lipids, and
3. Derived Lipids.

Simple Lipids

These lipids are esters of fatty acids with certain alcohols. These are sub classified according to the nature of alcohol.

a. *Fatty acids:* It consists of long chain of hydrocarbon having carbonyl group at one end and methyl group at another end. Fatty acids vary in chain length and degree of unsaturation. These are further classified as saturated fatty acids (without double bond in carbon chain) and unsaturated fatty acids (with double bond in carbon chain).

   In addition to straight chain compounds a number of branched chain and cyclic fatty acid, both, saturated and unsaturated are found.

   Triglycerides: These are neutral esters of glycerol and fatty acids, e.g. triscarin

b. *Waxes:* Waxes are esters or fatty acids with monohydric alcohol, e.g. spermaceti

c. *Isoprenoid lipids:* These are chemically unrelated to fats and phospholipids but their solubility are same.

Compound Lipids

These are esters of fatty acids with alcohols, containing additional groups. These are sub classified as—

a. *Phospholipids:* These lipids contain phosphorus as additional group, e.g. phosmalogen
b. *Glycolipid:* Combination of carbohydrate and lipids are glycolipids. These are found in chloroplast membranes.
c. *Lipoproteins:* Combination of lipid and protein are lipoprotein, e.g. cholestrol, glycerol

Derived Lipids

These are the lipids, which arise as a result of partial hydrolysis of lipid.

a. *Fatty acids:* These are hydrolysis products of fats and other lipids. Naturally occurring fats generally contain an even number of carbon atoms. These may be saturated or unsaturated, e.g. linoleic acid
b. *Steroids:* Steroids are naturally occurring cyclic compounds having a common structural base of cyclopentano perhydro-phenantherene ring. These have different physiological properities, e.g. testosterone

DETECTIVE TESTS FOR LIPIDS

1. *Iodine absorption test:* This test is for unsaturated fatty acids. A drop of iodine is added to fat solution (fat solution is prepared from chloroform) and shaken. The solution will be decolorize if unsaturated fatty acid is present.

2. *Rancidity:* When fat is allowed to stand for a sufficient length of time in contact with air and moisture in presence of light, it gets oxidized and becomes rancid.

3. *Emulsification:* When oil/fat is shaken with water; it is finally divided and dispersed in the water to form emulsion.

4. *Formation of acrolein:* Glycerol from fats dehydrated with the help of solid potassium bisulphate and acrylic aldehyde or acrolein is produced. It is noted by irritating odour.

LIPID METABOLISM

About 40 percent of the bodies caloric intake is derived from lipids and almost all of these calories come from fats, the triglycerols.

The use of fatty acids metabolism is predominantly performed in liver. The liver may play a modifying part in fat storage and retrieval. The major source of lipids entering the liver in free fatty acid form released from adipose tissue and transported in the systemic blood plasma complexed with albumin. Fatty acid oxidation yields twice the usable chemical energy that carbohydrates can deliver. As an example, 130 mols of ATP result from the oxidation of one mol of palmitic acid, as compared to 38 mols of ATP from one mol of glucose. On a weight basis, the caloric yield from fatty acids is about double that from carbohydrates; 9 kcal/g from fat vs. 4kcal/g from carbohydrate or protein).

The major aspects of lipid metabolism are involved with *Fatty Acid Oxidation* to produce energy or the synthesis of lipids, which is called Lipogenesis.

The sequence of reactions involved in the formation of lipids is known as Lipogenesis. Lipogenesis is not simply the reverse of the fatty acid spiral, but does start with acetyl-Co-A and does build up by the addition of two carbons units. The synthesis occurs in the cytoplasm in contrast to the degradation (oxidation), which occurs in the mitochondria. Many of the enzymes for the fatty acid synthesis are organized into a multienzyme complex called fatty acid synthetase.

The major points in the overall lipogenesis reactions are:

1. ATP is required.
2. The reactions are reductions (addition of H^+ and use of NADPH) which are the reverse of the oxidations in the fatty acid spiral.

Biosynthesis of fatty acids requires acetyl-Co-A as a key intermediate. It gets decarboxylated to malonyl Co-A. The acetyl-Co-A is first converted to citrate within mitochondrion by condensation with oxaloacetate. The citrate then passes out to cytoplasm and cleaved back to oxaloacetate and acetyl-Co-A with the consumption of ATP.

The first step in lipid metabolism is the hydrolysis of the lipid in the cytoplasm to produce glycerol and fatty acids. Free fatty acid enter mitochondria and undergo a process called β-oxidation for degradation to acetyl-Co-A. It is a five-step degradation reaction catalysed by a group of enzymes called fatty acid oxidase complex. With the use of ATP fatty acid gets converted to acetyl-Co-A

In mitochondria Acetyl-Co-A is converted to α-β unsaturated acyl-Co-A by reduction of FAD. A molecule of water is added to this to get β-hydroxy acyl-Co-A.

Next is again dehydrogenation step in presence of NAD to form β-keto-acyl-Co-A, which then splits into molecules of acyl-Co-A and acetyl-Co-A. The complex cycle results in generation of four ATP molecules.

DISEASES RELATED TO LIPID METABOLISM

1. *Steatorrhoea:* Maldigestion of fats due to inadequate secretion of pancreatic lipase or bile salts or even may be defective absorption due to intestinal diseases like coeliac disease results in excessive excretion of fat in feaces. This is called as Steatorrhoea
2. Obesity is another disorder due to accumulation of excess of body fat.
3. *Lipidosis:* This denotes the abnormal lipoproteins in blood or specific lipids in tissues.
4. *Hyperlipidemia:* This is a condition in which plasma cholesterol or plasma triglyceride level is increased. This condition occurs due to inherent genetic defects.

BIOCHEMICAL IMPORTANCE OF LIPID

Lipids are served as efficient source of energy when stored in adipose tissue and of great importance in biochemical processes. They are components of cell membrane and thus concerned with phenomenon of cell permeability and cell organization. Fat gives 9 cal of energy per gram on oxidation of CO_2 and water. In addition to providing flavors and satiety to diet, fat serve as vehicle for fat soluble vitamins A, D, E and K. Fats provide building blocks for different high molecular weight substances. Fatty acids form substances, which are essential for maintaining cellular integrity such as lipoproteins and glycolipids. Fats provide essential fatty acids, which are not synthesized by human body.

10 Nucleic Acids

Nucleic acid is a macromolecule with acid property. As it was isolated from nucleus of cells, it is called nucleic acid. It is made up of Carbon, Hydrogen, Oxygen, Nitrogen and Phosphorus.

Nucleic acid molecule is a long chain polymer. It is composed of monomeric units, called nucleotides. Each nucleotide consists of nucleoside and a phosphate group. Each nucleoside consists of a pentose sugar and a nitrogenous base. The sugar is ribose in case of RNA and deoxyribose in case of DNA.

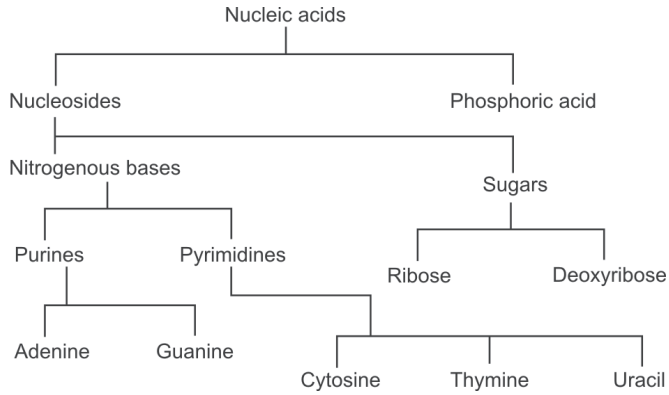
Nitrogenous bases are of two types namely—purine and pyrimidine. There are two main purine bases, adenine and guanine. Similarly there are three main pyrimidine bases. These are— cytosine, thymine and uracil. Cytosine and thymine are commonly found in DNA. Cytosine and uracil are found in RNA.

Nucleoside: A base combined with a sugar molecule is called nucleoside. In DNA, four different nucleosides are present. These are adenosine, guanosine, cytosine and thymine. In RNA deoxyribose sugar is replaced by ribose sugar and the base thymine is replaced by uracil.

Nucleotides: A nucleotide is derived from nucleoside by the addition of a molecule of phosphoric acid. A number of nucleotide units link with one another to form a polynucleotide chain.

STRUCTURE OF DNA

DNA molecules consists of two polynucleotide chains running in opposite directions and coiled in such a way that adenine of one strand is always in front of thymine of other strand. Guanine of one chain always faces cytosine in complementary strand. These pairs of bases



(A:T, G:C) are bonded by weak hydrogen bonds. This arrangement of bases is called base pairing rules and the overall structure is called as double helical structure (Fig. 10.1B).

The sugar present in DNA is called deoxyribose . It is a pentose sugar, which contains five carbon atoms ($C_5H_{10}O_4$). It contains one oxygen atom less than the ribose sugar.

In each DNA molecule, the deoxyribose sugar is attached to a phosphoric acid at one side and a nitrogenous base at other side. The phosphoric acid molecule is linked to sugar at carbon atom no. 3 or 5. The nitrogen base molecule is joined to the sugar by a glycosidic bond. This is formed between carbon atom no. 1 of deoxyribose and nitrogen atom 3 or 9 of nitrogen base. A phosphodiester bond joins two nucleotides. It is formed between the carbon atom no. 3 of sugar of one nucleotide and phosphate component at the 5th position of another nucleotide (Fig. 10.1A).

At one end of polynucleotide chain, third carbon of sugar is free called as 3' end and at the other end 5th carbon of sugar is free, called as 5' end. The 3' end of one chain lies close to 5' end of other chain. Hence two strands of DNA are anti parallel. The polynucleotide chains of DNA molecule are linked by nucleotides of adjacent chain.

In mammalian cells, DNA is always formed in nucleus where it exists in combination with basic proteins (histones) giving rise to structures, which are called chromosomes.

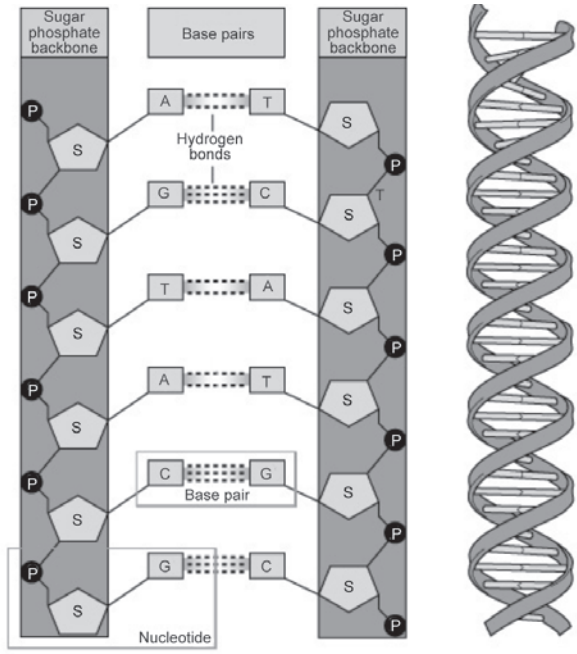


Fig. 10.1 A and B: Structure of DNA

STRUCTURE OF RNA

These are single stranded polynucleotides involved in the translation of genetic information contained in DNA. The pyrimidines present in RNA are Cytosine and Uracil. The number of purine and pyrimidine are not equal. Three types of functionally different RNAs are found in living cells.

1. *Messenger RNA(mRNA):* It is a single strand RNA of low molecular weight which is synthesized in the nucleus of mammalian cells. The sequence of nucleotides in mRNA is determined by the base-paring rule with one strand of DNA as a guiding polynucleotide. The mRNA thus formed has a nucleotide sequence similar to one strand of DNA, and complementary to the other strand the only difference being the replacement of thymine by uracil in RNA. This process in known as transcription of genetic information.

The mRNA thus formed carries the message for the synthesis of appropriate protein to the cytoplasm in the form of triplet code. There are one or more triplet codons for each amino acid. The sequence of nucleotides in mRNA, therefore determines the arrangement of amino acids in a polypeptide chain. There is separate mRNA for the synthesis of different proteins. The site of protein synthesis is ribosome. The process of transfer of information from mRNA in protein synthesis is called translation of genetic information. mRNAs are 3 to 5 percent of total RNA. These contain 500 to 1500 nucleotide.

2. *Ribosomal RNA(rRNA):* It is also a single stranded RNA synthesized in the same manner as the mRNA. However, it does not contain any genetic information. It is associated with the ribosome where it is found in combination with proteins. The main function of rRNA is to maintain the structural integrity of ribosome and to bind the mRNA, the tRNA and enzymes required in the protein synthesis. rRNAs are 80 percent of total RNA. These contain many nucleotides.

3. *Transfer RNA(tRNA):* It is low molecular weight RNA which is involved in the transfer of amino acids pool to the site of protein synthesis, i.e. ribosomes. For each amino acid there is a specific tRNA. tRNAs are 10 to 15 percent of total RNA. The molecule of tRNA is folded to attain a shape of cloverleaf. It contains 73 to 95 percent nucleotide.

BIOLOGICAL IMPORTANCE OF NUCLEIC ACID

1. The nucleic acids play an important role in preserving and transmitting the genetic properties of an organism.
2. The DNA stores the hereditary character of a cell in the form of sequence of nucleotides and transfers this information to daughter cells by replication.
3. Nucleic acids synthesize the various proteins of the protoplasm.
4. RNA is associated with memory storage functions in brain.
5. DNA produces mutations resulting in new characters.

11 Enzymes and Coenzymes

Enzymes are proteins that act as catalyst for biochemical reactions. Almost all biochemical reactions require catalyst. Enzyme increases the rate of reaction without being consumed in it. Enzymes are very specific in their action and thus separate enzymes exist for different reactions, with some exceptions.

The term enzyme was first introduced by Kuhne in 1878 (i.e. En = in and Zyme = Yeast). The enzymes are named by adding 'ase' to the name of substrate on which they act and/or the reaction they catalyse.

e.g.	Name of substrate	Enzyme
	Urea	Urease
	Maltose	Maltase

International enzyme commission (IEC) has classified enzymes into six classes based on the reaction of catalysis. Each class is subdivided into subclass according to the specifications of substrate, reactive group, co-enzyme, etc.

1. *Oxidoreductase:* These enzymes catalyse redox reactions, i.e. removal of hydrogen or addition of oxygen.

$$\text{Succinic acid} \xrightarrow{\text{Succinate dehydrogenase}} \text{Fumaric acid}$$

2. *Transferases:* These enzymes catalyse transfer reactions in which groups like $-NH_2$, $-CH_3$, $-CH_2OH$ are transferred from reactants to products.

$$\text{e.g. Alanine + Oxaloacetic acid} \xrightarrow{\text{Transaminase}} \text{Pyruvic acid + Aspartic acid}$$

4. *Hydrolyses:* These enzyme catalyse the hydrolytic cleavage reaction.

   e.g. Protein $\xrightarrow{\text{Protease}}$ Free amino acids

5. *Lyases:* These enzymes catalyse the non- hydrolytic cleavage reaction.
   e.g. aldolase, histidase, etc.
6. *Isomerases:* These are the enzymes, which are responsible for isomerization reaction.

   e.g. Dextrose $\xrightarrow{\text{Dextrose isomerase}}$ Fructose

7. *Ligases:* These enzymes catalyse the formation of bonds,
   e.g. synthetases

   On the basis of site of action, enzymes can be classified as

   a. Endoenzymes: Those who act only inside the cells are known as endoenzymes, e.g. isomerases
   b. Exoenzymes: Those, which are secreted outside the cell, are known as exoenzymes, e.g. lipases
   c. Constitute enzymes: The enzymes, which are produced in absence of the substrate, are known as constitute enzymes,
      e.g. enzymes of glycolytic series
   d. Induced enzymes: These are present in trace amounts but their concentration gets immediately increased in presence of substrate on which they act, e.g. ethanol, barbiturates are powerful in inducing hepatic microsomal enzymes.

FACTORS AFFECTING ENZYME REACTION

1. *Enzyme concentration:* Enzyme activity is directly proportional to the concentration of enzymes in the system. The enzymes are usually present in very low concentration.
2. *Effect of pH:* Most enzymes have a characteristic pH at which their activity is maximum. Going below and above this pH the activity declines.

3. *Effect of temperature:* The rate of enzyme catalysed reaction increases as the temperature increases up to optimum temperature. After that further rise in temperature decreases the velocity of reaction.
4. *Effect of inhibitors:* Presence of inhibitors reduces the enzyme action, e.g. heavy metals are enzyme inhibitor.
5. *Enzyme activators:* Presence of activators in certain concentration increases the enzyme activity, e.g. Presence of cysteine HCl increases proteolytic activity.

The clinically important enzymes are:
1. Alkaline phosphatases
2. Acid phosphatases
3. Amylase
4. Transminases (SGOT and SGPT)
5. Lactic dehydrogenase

COENZYMES AND COFACTORS

Coenzymes are non-protein dialyzable organic compounds, which are non-covalently bound to the enzyme protein and are responsible for the catalytic activity of several enzymes. Coenzymes are generally derived from the water-soluble B-complex vitamins and carry out transfer of hydrogen or any other group of substrate in an enzymic reaction. The following table gives a list of various coenzymes with their functions and origin.

Coenzyme	*Function*	*Corresponding vitamin B*
NAD^+ NADP	Transfer of hydrogen	Nicotinamide
FAD, FMN	Transfer of hydrogen	Riboflavin
ATP	Transfer of phosphate group	——
UDP	Transfer of sugar	——
Biotin	Required in carboxylations	Biotin
CoA	Transfer of acyl group	Pantothenic acid
TPP	Transfer of acetaldehyde	Thiamine
Pyridoxal phosphate	Transfer of amino group	Pyridoxine

Coenzymes function as acceptors or donors of specific group, or the hydrogen atoms or electrons from or to the substrate. Regeneration of the catalytically active coenzyme occurs either in the same reaction

or in a subsequent metabolic reaction. In most of the enzymatic reactions, where the coenzyme acts as a substrate, there is an ordered sequence of addition of various reactants. The enzyme first binds to the coenzyme molecule followed by binding of substrate. After the enzymatic transfer of concerned group/ electron has occurred, the coenzyme product is first released, followed by release of the reaction products.

Cofactors include metal ions like Mg^{++}, Mn^{++},Ca^{++}. The active enzyme cofactor complex is termed holozyme while enzyme alone is called apozyme. Following is the list of some enzymes and their metal ion cofactors.

Enzyme	*Cofactor*
Alcohol dehydrogenase	Zn^{++}
Arginase	Mn^{++}
Tyrosinase	Cu^{++}.
Cytochromes	Fe^{++} / Fe^{++} $^{+}$
Phosphohydrolases	Mg^{++}
Plasma membrane ATPase	Na^{+}

12 Water and Mineral Metabolism

DISTRIBUTION OF WATER IN BODY

The average body water content is 60-70 percent of the body weight. In adult male of 70 kg the total body water is about 40 liters. The amount of water in females is less than in males. The distribution of water in the body as—

1. Intracellular — 50 percent of the body weight.
2. Extracellular — 20 percent of the body weight. It can be further divided as—
    a. Plasma—5.5 percent of the body weight.
    b. Intestinal and lymph fluids— 80 percent
    c. Dense connective tissue, cartilage and bone — 6.5 percent
    d. Transcellular fluids (aqueous and vitreous humour, CSF, endolymph; etc.) —5 percent

Water Intake—Water is supplied to the body by the following processes:

a. Dietary liquids
b. Solid foods
c. Oxidation of foodstuffs: It is obtained from the combustion of fats, proteins and carbohydrates. The oxidation of fats yields 107 ml/100 gm, proteins 41 ml/100 gm and carbohydrates 56 ml/100 gm.

Water output: Water is lost from the body by the following routs:

a. Urine
b. Respiration
c. Lactation
d. Faeces

e. Evaporation from skin and lungs
f. Eyes (tears)

FUNCTIONS OF WATER

1. *Solvent:* One of the most important properties of water is its capacity to dissolve different kinds of substances. It is therefore the most suitable solvent for cellular components. Water brings together various substances in contact when chemical reactions take place.
2. *Catalytic action:* Water accelerates a large number of chemical reactions in the body due to its ionizing power.
3. *Lubricating actions:* Water acts as a lubricant in the body and prevents friction in joints, pleura, conjunctiva, and peritoneum.
4. *Heat regulation:* By virtue of its high specific heat, water prevents any significant rise in the body temperature due to heat liberated from body reactions. The loss of heat from the body is also regulated by the evaporation of water from skin and lungs and its removal in urine.
5. *Water balance:* Equilibrium is maintained between the intake and the loss of water from the body. The regulatory mechanism of the body water is influenced in addition to other factors by certain hormones, such as ADH,vasopressin, oxytocin, and aldosterone.

The balance sheet of water intake and loss is given as:

Water intake			*Water loss*		
Drinks	48 %	1350 ml	Lungs	12%	500 ml
Solid	40 %	900 ml	Skin	24%	700 ml
Oxidation of food	12%	450 ml	Urine	56%	1400 ml
			Faeces	08%	100 ml
	100%	2700 ml		100%	2700 ml

MINERALS

Minerals are inorganic substances mined from the earth. They are not of plant or animal origin. They exist naturally on and in the earth and many are critical parts of human tissue and are termed “essential” nutrients. Of the 92 naturally occurring elements, the 14 minerals that

have been shown by research to be essential to human health are: calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc. Essential macrominerals are those needed in significant quantities (such as calcium) – usually measured in milligrams, and essential trace minerals are those needed in minute quantities (such as selenium) – usually measured in micrograms (one microgram [mcg] equals 1/1,000th of a milligram [mg]).

Minerals are required for a variety of physiological functions. Their functions are—

1. Maintenance of osmotic pressure of cell
2. Transport of oxygen
3. Growth and maintenance of tissues and bones
4. Working of nervous system
5. Muscle contraction
6. Maintenance of electrolytic balance
7. Acid-base balance

The major elements that compose the human body and their relative amounts are as follows:

Mineral content of human Element body	*Percent*	*Approximate amount (in gm) in 70 Kg adult*
Ca^{++}	1.50	1050
P	1.00	700
K^{+}	0.35	245
Na^{++}	0.15	105
Cl^{-}	0.15	105
Mg^{++}	0.05	035
Fe^{++}	0.004	003
Zn^{++}	0.0033	02.3

The Important Minerals Required in Human Body

1. **Iron:** Iron is an essential constituent of haemoglobin and certain enzymes such as cytochrome oxidase, catalase and peroxidase. It performs two important functions in the body—to transport oxygen to tissues (through Hb) and to take part in oxidation-reduction reactions (cytochrome system).

Sources: meat, liver, eggs, spinach and fruits.

Absorption: Dietary intake of iron is mainly in ferric (Fe^{+++}) form as hydroxides or in organic compounds. The action of gastric HCl and of some organic acids liberates free ferric ions, which in turn are reduced to ferrous ions (Fe^{++}) by reducing substances such as cysteine or ascorbic acid. The ferrous form of iron is more soluble and thus easily absorbed. The absorption of iron occurs in duodenum and stomach.

Transport and storage: Iron is transported in plasma in ferric form, which remains firmly bound to a specific β-globulin,transferring. The normal concentration of protein bound iron in plasma is 50 - 180μ gm/ 100ml.

Iron is stored chiefly in mucosal cells of intestine, liver, spleen and bone marrow as ferritin.

Daily requirement: Infants – 6–15 mg, Children- 10–18 mg, Adult(male) 10 mg, female- 18 mg.

2. **Calcium:** Calcium is the most important and essential mineral. It performs following functions — Builds and maintains bones and teeth; regulates heart rhythm; eases insomnia; helps regulate the passage of nutrients in and out of the cell walls; assists in normal blood clotting; helps maintain proper nerve and muscle function; lowers blood pressure; important to normal kidney function and in current medical research reduces the incidence of colon cancer, and reduces blood cholesterol levels.

   Sources: Good sources of calcium include milk and milk products, yogurt, ricotta, cheese, oysters, salmon, collard greens, spinach, ice cream, cottage cheese, kale, broccoli and oranges.

   Absorption: Calcium is taken in diet as calcium phosphate, carbonate tartarate and oxlate. Its absorption occurs mainly in intestine.

   Deficiency: May result in arm and leg muscles spasms, softening of bones, back and leg cramps, brittle bones, rickets, poor growth, osteoporosis (a deterioration of the bones), tooth decay, depression.

Toxicity: Occurs in hypervitaminosis D and hyperthyroidism or idiopathic hypercalcemia. It is characterized by vomiting, abdominal cramps, nephrocalcinosis.

3. **Phosphorus:** Phosphorus is widely distributed in the body. It has following functions: Builds and maintains bones and teeth along with calcium, required for the synthesis of phospholipids, nucleotides, phosphoproteins, organic phosphates and energy rich compounds(ATP)

   Sources: Food sources of phosphorus include protein-rich foods such as meats and dairy products, although some is present in almost all foods.

   Absorption: The absorption of phosphorus is intimately related to calcium absorption. High calcium diet diminishes phosphorus absorption due to the formation of insoluble calcium phosphate.

   In blood, phosphorus occurs in three forms—a. Inorganic phosphate (2-5 mg/100 ml) b. Organic phosphorus (14-29 mg/100 ml) c. Phospholipids (8-18 mg/100 ml). Serum levels are regulated by kidney reabsorption.

   In rickets, serum inorganic phosphate level comes down to about 1-2 mg/100 ml. People taking aluminum hydroxide as an antacid for extended periods of time may develop a phosphorus deficiency since the aluminium prevents phosphorus absorption.

4. **Sodium:** It is a principal cation in extracellular fluid. It regulates plasma volume, acid-base balance, nerve and muscle function and Na^+ / K^+ ATPase.

   Source: The salt added to prepare food is the main source of sodium.

   Metabolism: Metabolism is regulated by aldosterone (progesterone, ACH)

   Deficiency: Deficiency is related to diarrhea, vomiting, Addison's disease, excessive sweating, over use of diuretics, salt loosing nephritis, diabetes mellitus. It is characterized by headache, nausea, cramps, fall in BP, oliguria, increased pulse rate.

Toxicity: Occurs in cardiac failure, hepatic cirrhosis, acute glomerulonephritis, premenstrual phase of cycle, excess of ACTH, ACH, testosterone. In hypersensitive individuals it causes elevation of blood pressure and oedema.
Plasma level: 135 – 145 mEq/lit.
Daily requirement: 5 – 15 gm.

5. **Potassium:** Works with sodium to regulate the body's waste balance and normalize heart rhythms; aids in clear thinking by sending oxygen to the brain; preserves proper alkalinity of body fluids; stimulates the kidneys to eliminate poisonous body wastes; assists in reducing high blood pressure; promotes healthy skin.

   Sources: Potassium is widely available in foods, but mostly in unprocessed fresh foods – especially fruits and vegetables and nuts.

   Metabolism: Metabolism is regulated by aldosterone

   Deficiency: May result in poor reflexes, nervous disorders, respiratory failure, cardiac arrest, muscle damage.

   Toxicity: Excess causes cardiac arrest, small bowl ulcers. It is a feature of diabetic ketoacidosis, severe burn, blood loss, acute renal failure, Addison's disease, chronic renal disease.

   Plasma level: 3 – 5 mEq/lit.

   Daily requirement: 4 gm.

6. **Magnesium:** Plays an important role in regulating the neuromuscular activity of the heart; maintains normal heart rhythm; necessary for proper calcium and Vitamin C metabolism; converts blood sugar into energy.

   Source: Good food sources of magnesium include seeds, unrefined grains, beans and other vegetables.

   Deficiency: Deficiency may result in calcium depletion, heart spasms, nervousness, muscular excitability, confusion; kidney stones.

   Toxicity: Depressed deep tendon reflexes and respiration.

7. **Copper:** It is necessary for the absorption and utilization of iron; helps oxidize Vitamin C and works with Vitamin C to form Elastin,

a chief component of the Elastin muscle fibers throughout the body; aids in the formation of red blood cells; helps proper bone formation and maintenance.

Sources: Copper is commonly found in whole grains, nuts, shellfish, liver and dark green, leafy vegetables.

Deficiency: May result in general weakness, impaired respiration, skin sores.

Toxicity: It is rare. Secondary to Wilson's disease characterized by more urinary excretion of copper and less serum copper.

8. **Iodine:** Aids in the development and functioning of the thyroid gland; regulates the body's production of energy; helps burn excess fat by stimulating the rate of metabolism; mentality, speech, the condition of the hair, skin, and teeth are dependent upon a well-functioning thyroid gland.

   Source: Iodized salt and sea food is the most common source of this essential trace mineral

   Deficiency: May result in an enlarged thyroid gland, slow mental reaction, dry skin and hair, weight gain, loss of physical and mental vigor.

   Toxicity causes thyrotoxicosis and goiter.

9. **Manganese:** An antioxidant nutrient; important in the blood breakdown of amino acids and the production of energy; necessary for the metabolism of Vitamin B_1 and Vitamin E; Activates various enzymes which are important for proper digestion and utilization of foods; is a catalyst in the breakdown of fats and cholesterol; helps nourish the nerves and brain; necessary for normal skeletal development; maintains sex hormone production.

   Source: Grains and cereal products are the best food sources of manganese.

   Deficiency: May result in paralysis, convulsions, dizziness, ataxia, loss of hearing, digestive problems, blindness and deafness in infants.

   Toxicity due to inhalation causes psychotic symptoms and Parkinsonism.

10. **Zinc:** Is an antioxidant nutrient; necessary for protein synthesis; wound healing; vital for the development of the reproductive organs, prostate functions and male hormone activity; it governs the contractility of muscles; important for blood stability; maintains the body's alkaline balance; helps in normal tissue function; aids in the digestion and metabolism of phosphorus.

    Sources: Meats, fish, beans, whole grains, pumpkin seeds, mushrooms and brewer's yeast are good food sources of zinc.

    Deficiency: May result in delayed sexual maturity, prolonged healing wounds, white spots on finger nails, retarded growth, stretch marks, fatigue, decreased alertness, susceptibility to infections.

    Too much zinc can lower copper retention, lower HDL ("good") cholesterol, gastrointestinal irritation, and vomiting.

11. **Molybdenum:** Molybdenum is involved in the operation of several key enzymes in the body. Readily available throughout the diet, deficiencies of this essential mineral are unusual, although rare deficiencies occur in people who suffer from malabsorption conditions. Milk, beans, cereals and bread are common food sources of molybdenum. Elevated levels of molybdenum can cause a loss of copper.

12. **Selenium:** A major antioxidant nutrient, protects cell membranes and prevents free radical generation thereby decreasing the risk of cancer and disease of the heart and blood vessels. Medical surveys show that increased selenium intake decreases the risk of breast, colon, lung and prostate cancer. Selenium also preserves tissue elasticity; slows down the aging and hardening of tissues through oxidation; helps in the treatment and prevention of dandruff.

    Source: Seafood and organ meats such as liver and kidney are high in selenium, whereas selenium levels in grains and vegetables vary widely, depending on local soil content.

    Deficiency: May result in premature aging, heart disease, dandruff, and loose skin. No more than 200 mcg of selenium daily is recommended for general use, because of possible toxicity. Excessive intakes of selenium can affect the

functioning of enzymes and normal bone and cartilage development, selenium in excess can cause nausea, loss of hair and nails, skin abnormalities and nerve damage.

13. **Chromium:** Works with insulin in the metabolism of sugar and stabilizes blood sugar levels; cleans the arteries by reducing Cholesterol and Triglyceride levels; helps transport amino acids to where the body needs them; helps control the appetite; medical research has shown that persons with low levels of Chromium in their bodies are more susceptible to having cancer and heart problems and becoming diabetic.

    Source: The only common food source is brewer's yeast.

    Deficiency: May result in glucose intolerance in diabetics; arteriosclerosis, heart disease, depressed growth, obesity, tiredness

    Chromium should not be taken in excess however—there have been reported cases of toxicity when used in high doses (>800 mcg/day).

TRACE MINERALS

Minerals that occur in tiny amounts or traces. These are cobalt, fluorine, iodine chromium, selenium, manganese, and molybdenum. They play a major role in health, since even minute portions of them can powerfully affect health. They are essential in the assimilation and utilization of vitamins and other nutrients. They aid in digestion and provide the catalyst for many hormones, enzymes and essential body functions and reactions. They also aid in replacing electrolytes lost through heavy perspiration or extended diarrhea and protects against toxic reaction and heavy metal poisoning.

13 Inborn Errors of Metabolism

Inborn errors of metabolism is a term applied to disorder, which arises, as a result of congenital metabolic defects. Such disorders are in most cases familial and occur due to deficiency of an enzyme in any particular metabolic pathway.

Some of the important diseases belonging to this category are described below.

CARBOHYDRATE METABOLISM

Glycogen Storage Disease (Glycogenosis)

It is a group of congenital disorders which occur due to deposition of large amounts of glycogen in several tissues such as liver, kidney, heart and muscle. Glycogen accumulation in these tissues occurs due to deficiency or lack of enzymes involved in the breakdown of glycogen. On the basis of the deficiency of various enzymes in different tissues, such disorders have been divided into following types:

Type 1 (Von Gierke's Disease)

Glycogen deposition predominantly occurs in liver and kidney. The disease is due to the deficiency or complete absence of glucose-6-phosphatase enzyme. This means that glycogen cannot be broken down to glucose completely. The level of glucose phosphate in such a situation favors glycogen synthesis. Since glucose cannot be derived from glycogen in this condition, children with this disease develop hypoglycemia. Thus, most of the energy requirements of the body are met by fat breakdown and this leads to ketosis and hyperlipemia. Children suffering from this disease are generally known to die young, though a number of them survive to adolescence.

Type 2 (Pompe's Disease)

In this condition, nearly all tissues contain abnormally large amounts of glycogen. The heart is generally found to be enlarged and there is weakness of muscles. The deficient enzyme in this case is acid maltase (α-1, 4-glucosidase) which destroys glycogen entering the lysosomes. Death usually occurs in infancy and in many cases within a year after birth.

Type 3 (Limit Dextrinosis)

Large amounts of limit dextrins accumulate in liver and muscle in this type of disease. The deficient enzyme in this case is debranching enzyme. Thus, further breakdown of glycogen after phosphorylase action is affected. The persons with limit dextrinosis are known to survive well into adult life.

Type 4 (Amylopectinosis)

In this condition large amounts of amylopectin accumulate mainly in liver due to the deficiency of branching enzyme. Hepatomegaly, cirrhosis and fasting hypoglycemia are some clinical manifestations of this disease. It is fatal and children die by 4 years of age.

Type 5 (McArdle's Disease)

This disease occurs due to the deficiency of muscle phosphorylase. Persons suffering from this disease do not tolerate even the moderate exercise. Although, high amounts of muscle glycogen are present, no lactate is detected in blood after exercise.

Type 6 (Her's Disease)

It is an ill-defined condition in which liver glycogen content is elevated due to the mild deficiency of liver phosphorylase. Clinically it is mild type 1 glycogenosis.

Type 7

In this condition there is deficiency of Phosphofructokinase enzyme which causes moderate accumulation of glycogen in skeletal muscle.

Glucose-6-phosphate and fructose-6-phosphate are also found to accumulate in this disease. Clinically the picture resembles with that of Type 5 glycogenosis.

Of the above-mentioned types of glycogen storage disease (glycogenosis) the most commonly occurring types are Type 1, 2, 3 and 6.

Pentosuria—Appearance of pentose sugar in urine is called Pentosuria. It may temporarily occur in normal person as a result of ingestion of foods containing high amounts of pentose such as fruits (plums, cherries and grapes). However, congenital defect in the enzyme catalyzing the conversion of L-Xyulose to L-Xylitol in the uronic acid pathway in liver, causes Essential Pentosuria (Chronic Pentosuria) In this condition L-Xylulose is excreted in urine irrespective of amount ingested.

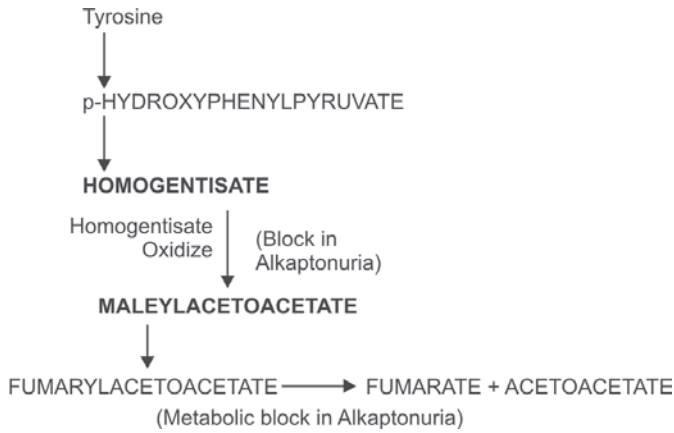
Galactosuria—This is hereditary disorder due to metabolic defect in the conversion of galactose to glucose. It occurs due to deficiency or complete lack of enzyme galactose-1-phosphate uridyltransferase which converts galactose-1-phosphate to glucose-1-phosphate. This results in accumulation of galactose-1-phosphate in tissues and blood. Infants with this condition suffer from malnutrition and wasting, mental retardation, galactosuria and eventually hepatomegaly and cirrhosis may also develop. Increased level of galactose reduces blood glucose concentration. On removal of milk from the food, however, the clinical symptoms of this disorder can be removed.

PROTEIN METABOLISM

Alkaptonuria

Alkaptonuria is a congenital metabolic disorder associated with the abnormal metabolism of tyrosine. It is characterized by the presence of large amounts of homogentisate in urine, which turns black due to oxidation in air. Alkaptonuria is caused by the absence of homogentisate oxidase which catalyses the conversion of homogentisate to maleylacetoacetate which leads to the formation of fumarate and acetoacetate in the body as follows:

Flow chart 13.1: Pathway of alkaptonuria

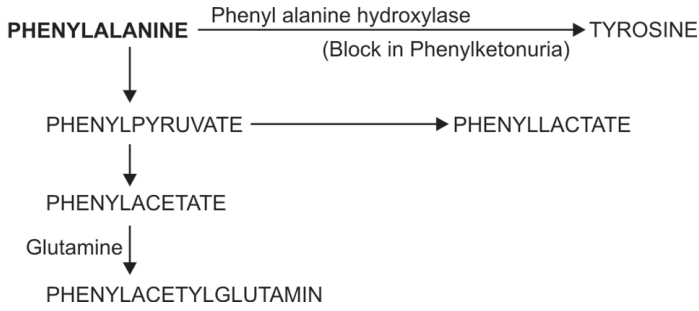


Although large amounts of homogentisate are excreted in urine daily, Alkaptonuria does not produce any important clinical manifestation. However, in some cases, darkening of cartilage and tendons (orchronosis) has been observed. This may occur due to the deposition of oxidized homogentisate.

PHENYLKETONURIA

Phenylketonuria is a congenital metabolic disorder, which is characterized by the excretion in urine of large amounts of phenylalanine and phenylpyruvate with good amounts of phenyllactate, phenylacetate and phenylacetylglutamin.

Flow chart 13.2: Pathway of phenylketonuria



Phenylketonuria occurs due to the absence of the enzyme phenylalanine hydroxylase which catalyses the conversion of phenylalanine to tyrosine in human liver. In this condition, phenylalanine is metabolized through an alternate pathway, which results in the formation and excretion of large quantities of its pyruvate and acetate derivative as above.

A prominent manifestation of Phenylketonuria is mental retardation, which probably occurs due to the accumulation of phenylalanine and its toxic metabolites in the body. Mental deficiency, however, can be avoided by feeding phenylketonuric children with less phenylalanine containing diets. Due to certain secondary effects of Phenylketonuria on tyrosine metabolism, the formation of melanin is also inhibited which leads to diminished pigmentation of skin and hair.

LIPID METABOLISM

Accumulation of abnormal quantities of certain lipids characterizes some disease (lipidosis) which occur due to metabolic defect in lipid metabolism.

Niemann-Pick disease—In this condition, abnormal amounts of sphingomyelin accumulate in spleen, brain and liver. It is due to the deficiency of enzyme called sphingomylinase in the metabolism of sphingomyelin. It is an inherited disease of infancy and leads to death within the first two years of life. Clinically it is manifested as anaemia and leukocytosis.

Tay-Sachs' disease—In this disease-increased concentrations of gangliosides (GM_2) are found in brain and spleen. The defect is caused by the deficiency of Hexosaminidase. A enzyme in tissues.

Gaucher's disease—In this condition large amounts of cerebrosides (Glucosyl-Ceramide) deposit in spleen, liver, lymph, nodes and bones. This defect occurs due to the deficiency of an enzyme called glucosyl ceramide hydrolase.

NUCLEIC ACID METABOLISM

Increased purine synthesis with hyperuricemia occurs in several hereditary metabolic diseases.

Lesch-Nyhan syndrome—This condition is characterized by the presence of large amounts of uric acid in urine. It leads to a severe neurological syndrome accompanied by gout in childhood. There is complete deficiency of phosphoribosyl transferase. This disorder is transmitted to offsprings through X-chromosomes.

Hereditary xanthinuria—It is a rare genetic disorder caused by the deficiency of xanthine oxidase. Thus, large amounts of xanthine with lesser quantities of hypoxanthine is excreted in urine. Uric acid levels in blood as well as in urine are found to be very low.

14 Hormones

The endocrine system consists of a number of separate glands situated in various parts of body. Their secretions are called hormones. The glands are ductless glands and their secretion is released directly into the blood stream. The organ on which the hormone acts is called as target organ. The hormones are carried away from gland to the target organ through blood. Hormones act as chemical regulators. They are specific in their action so that one hormone can regulate only a particular metabolic process in a particular organ. Chemically a hormone may be protein, steroid or amines.

Following are the hormones present in man:

GROWTH HORMONE (GH) OR SOMATOTROPHIC HORMONE (STH)

Growth hormone is secreted by anterior lobe of pituitary gland. It is protein in nature. It stimulates the multiplication of cells and increases body growth. It increases the secretion of milk during lactation. STH has a remarkable effect on metabolism. It increases protein synthesis and release of fatty acids from the adipose tissue. STH decreases the utilization of carbohydrate for energy and stimulates the storage of carbohydrates. It increases intestinal absorption of calcium as well as its excretion. In addition to calcium, sodium, potassium, magnesium, phosphate and chloride ions are also retained.

ADENOCORTICOTROPHIC HORMONE (ACTH)

ACTH is a protein hormone, secreted by anterior lobe of pituitary gland. It stimulates the activity of adrenal cortex, including the secretion

of glucocorticoids. Hence, ACTH is necessary to counteract the psychological and physical stress situations. Its deficiency causes rheumatoid fever, Addison's disease etc.

THYROTROPHIC HORMONE/THYROID STIMULATING HORMONE

It is a protein hormone secreted by anterior lobe of pituitary gland. This hormone stimulates the growth and activity of thyroid gland, which is essential for normal life. This increases the thyroxine secretion.

FOLLICLE STIMULATING HORMONE (FSH)

It is a protein hormone secreted by anterior lobe of pituitary gland. In females, it increases the number and size of the Graffian follicles. In males it stimulates the testis for spermatogeneis.

LUTEINISING HORMONE(LH)

It is glycoprotein hormone secreted by the anterior lobe of pituitary gland. It makes the Graffian follicle to grow and secrete another sex hormone called oestrogen. In co-operation with FSH, it causes the rupture of follicle and ovulation. LH causes appearance, growth and persistence of corpus luteum in the ovary. In male, LH stimulates the interstitial cells of testis and consequently the production of androgen.

MELANOCYTE STIMULATING HORMONE

It is a polypeptide hormone secreted by pars distalis. It affects the melanocytes or pigment cells leading to darkening of skin.

VASOPRESSIN OR ANTIDIURETIC HORMONE (ADH)

It is a protein hormone secreted by posterior lobe of pituitary gland. It constricts arterioles and capillaries, causing the raise of blood pressure. It stimulates the nephron to reabsorb water from urine and thus reduces volume of urine formed. Deficiency of this hormone produces large volume of very diluted urine, this defect is called diabetes insipidus.

OXYTOCIN / PITOCIN

It is a protein hormone secreted by neurohypophysis. It promotes the fertilization of ovum. It causes the contraction of mammary glands resulting in the ejection of milk. Another effect of oxytocin is to produce contractions of uterus, especially in pregnant mothers.

THYROXIN

It is a protein hormone that contains iodine. It is secreted by thyroid gland. According to the number of iodine atoms per molecule, there are two forms of thyroid hormone called triiodothyronine (T_3) or tetraiodothyronine (T_4). The colloid inside the follicles contains these hormones in a bound form called thyroglobulin. This is a storage form of hormone. T_4 is produced more in quantity than T_3, but T_3 is physiologically more active and long lasting. The effects of T_3 and T_4 are similar. These include—

a. They increase overall metabolism of body and the protein synthesis.
b. They increase fat degradation and thus contribute to decrease in blood cholesterol level.
c. They have effect on sympathetic nerves, there by increase heart rate, respiratory rate, digestive secretions, etc.

Both the excess and insufficient production of thyroid hormones leads to severe abnormalities. These are referred to as hyperthyroidism and hypothyroidism respectively.

THYROCALCITONIN

It is a protein hormone secreted by thyroid gland. It acts on bones and kidneys to reduce the level of calcium in blood.

PARATHORMONE (PTH)

It is a protein hormone. It increases calcium level and also control intracellular disposition of phosphate. It also helps to metabolize vitamin D.

GLUCOCORTICOIDS

These are a group of steroid hormones secreted by adrenal cortex. These are concerned with carbohydrate metabolism. It stimulates the

formation of glycogen in the liver. Over secretion of glucocorticoids leads to Cushing's syndrome.

MINERALOCORTICOIDS

These are steroid hormones secreted by adrenal cortex. The main function of these hormones is concerned with metabolism of minerals. They help to reabsorb NaCl and water from the renal tubules. They cause increased excretion of potassium. Deficiency of mineralocorticoid leads to Addison's disease.

ADRENALINE / EPINEPHRINE

It is a protein hormone secreted by adrenal medulla. It is produced and effective under emergency conditions such as stress, emotion, threatened dangers, etc. It promotes glycogenolysis, oxygen consumptions, sweating, respiratory rate, rate of heartbeat and cardiac out put. It constricts the veins and arteries of the skin. The blood pressure rises sharply.

NORADERENALINE/NOREPINEPHRINE

It is a protein hormone secreted by adrenal medulla. It is also secreted under emergency condition. Most of its actions are similar to those of adrenaline but some are just reverse of adrenaline.

INSULIN

It is a polypeptide, secreted by islets of Langerhans. The main role of insulin is to lower the blood sugar level. Deficiency of insulin causes diabetes mellitus.

GLUCAGON

It is a polypeptide hormone secreted by islets of Langerhans. It works opposite to insulin. It accelerates the synthesis of glucose from fats and amino acids.

ANDROGEN

It is a sterol, secreted by testis, adrenal cortex, ovary and placenta. It brings about development of male genitalia. It causes the growth of

accessory male sex organs and development of secondary sex character. It causes spermatogenesis and also responsible for sexual behavior of man. Baldness of man is brought out by androgen; it also increases the thickness of skin.

ESTROGEN

It is a steroid produced by adrenal cortex, testes, placenta and pregnancy urine is very rich source of estrogen. It is responsible for all the puberty changes and secondary sexual characters in female.

PROGESTERONE

It is a steroid secreted by Corpus Luteum of ovary, placenta and adrenal cortex. It is responsible for premenstrual changes in urine. It thus helps in implantation of the fertilized ovum. It helps in lactation, enlargement of birth canal. It also helps to retain salt and water.

CHORIONIC GONADOTROPHIN (CG)

It is a glycoprotein found in placenta and urine of pregnant women. It aids in maintenance of Corpus luteum, and stimulates corpus luteum to secrete oestrogen and progesterone.

CHORIONIC GROWTH HORMONE

It is a protein hormone secreted by the placenta from the first few weeks of pregnancy. It stimulates glandular elements of the mammary glands.

MELATONIN

It is a protein hormone released by pineal body. It inhibits the activities of the ovary.

15 Diabetic Profile

Diabetes is a disease in which blood glucose levels are above normal. People with diabetes have problems converting food to energy. After a meal, food is broken down into a sugar called glucose, which is carried by the blood to cells throughout the body. Cells use insulin, a hormone made in the pancreas, to help them convert blood glucose into energy.

People develop diabetes because, the pancreas does not make enough insulin or because, the cells in the muscles, liver, and fat do not use insulin properly, or both. As a result, the amount of glucose in the blood increases while the cells are starved of energy. Over the years, high blood glucose, also called hyperglycemia, damages nerves and blood vessels, which can lead to complications such as heart disease and stroke, kidney disease, blindness, nerve problems, gum infections, and amputation.

TYPES OF DIABETES

The three main types of diabetes are type 1, type 2, and gestational diabetes.

Type 1/Juvenile Diabetes/Insulin-dependent Diabetes Mellitus/IDDM

- Type 1 diabetes, formerly called juvenile diabetes, is usually first diagnosed in children, teenagers, or young adults. In this form of diabetes, the beta cells of the pancreas no longer make insulin because the body's immune system has attacked and destroyed them.

Type 2 / Adult-onset Diabetes/Noninsulin-dependent Diabetes Mellitus/NIDDM

- Type 2 diabetes, formerly called adult-onset diabetes, is the most common form. People can develop it at any age, even during childhood. This form of diabetes usually begins with insulin resistance, a condition in which muscle, liver, and fat cells do not use insulin properly. At first, the pancreas keeps up with the added demand by producing more insulin. In time, however, it loses the ability to secrete enough insulin in response to meals.
- Gestational diabetes, develops in some women during the late stages of pregnancy. Although this form of diabetes usually goes away after the baby is born, a woman who has had it is more likely to develop type 2 diabetes later in life. Gestational diabetes is caused by the hormones of pregnancy or by a shortage of insulin.
- Pre-diabetes, in pre-diabetes, blood glucose levels are higher than normal but not high enough to be characterized as diabetes. However, many people with pre-diabetes develop type 2 diabetes within 10 years. Pre-diabetes also increases the risk of heart disease and stroke. With modest weight loss and moderate physical activity, people with pre-diabetes can delay or prevent type 2 diabetes.

DIAGNOSIS OF DIABETES

The following tests are used for diagnosis:

- *A fasting plasma glucose test* measures the blood glucose after at least 8 hours of fasting. This test is used to detect diabetes or pre-diabetes.
- *A post meal plasma glucose test* measures the blood glucose after usual adequate meals. Patient must complete meal within 15-20 min. Specimen is collected at 2 hr from beginning of meal.
- *An oral glucose tolerance test* measures the blood glucose after at least 8 hours of fasting and 2 hours after drinking a glucose-containing beverage. This test can be used to diagnose diabetes or pre-diabetes.
- *In a random plasma glucose test,* blood glucose can be checked without regard to when one ate the last meal. This test, along with an assessment of symptoms, is used to diagnose diabetes but not pre-diabetes.

Positive test results should be confirmed by repeating the fasting plasma glucose test or the oral glucose tolerance test on a different day.

Blood Glucose

Blood glucose can be determined at different times. As stated above, it may be fasting, post meal or random.

Specimen: Sreum, plasma, or whole blood can be used. Serum should be separated from blood clot within half hr of blood collection. For plasma preparation, fluoride with oxalate is used as an anticoagulant.

There are three important methods to determine plasma glucose level.

I. Folin-Wu method
II. O-Toludine method
III. Glucose oxidase method

Folin-Wu Method

It is economical, simple and convenient method.

Principle

Glucose on boiling with alkaline copper solution reduces copper from the cupric to cuprous state. The cuprous oxide so formed reduces phosphomolybdic acid to blue coloured molybdenum blue, which is measured colorimetrically. The intensity of blue colour is proportional to glucose concentration.

Reagents

1. 10 percent Sodium tungstate
2. 0.66 N sulphuric acid
3. Alkaline copper sulphate solution
4. Phosphomolybdate solution
5. Stock glucose standard
6. Working standard—dilute 1ml of glucose stock standard with 9 ml of distill water to give 100 mg percent working standard. Prepare this everyday.

Procedure

Part I: Preparation of Protein Free Filtrate

In a 10 ml test tube take 3.5 ml distill water and 0.1 ml of plasma / serum, followed by 0.2 ml of 10% sodium tungstate. Mix and add slowly 0.2ml of 2 / 3 N sulfuric acid. Mix well stand for 5 min. Filter or centrifuge.

Part II: Colour Development

Folin blood sugar test tubes are recommended for this test. The tubes are specially designed to prevent contact of atmospheric oxygen with the reaction mixture, which affects the result.

Fig. 15.1: Folin Wu's blood sugar tube

1. Take four Folin-Wu tubes (Fig. 15.1) and label it as blank (B), Standard (S), Control (C) and Test (T). Add 1ml of distilled water, glucose standard, protein free filtrate of control serum and test serum in respective tubes.
2. Add 1ml of alkaline copper reagent to each tube.
3. Transfer the tubes to boiling water bath for 10 min.
4. Cool the tubes for 2-3 min. under running tap water without shaking
5. After cooling add 2 ml phosphomolybdate reagent to each tube.
6. Mix well by inversion and dilute the contents of each tube upto 12.5 ml with distilled water.
7. Set the wavelength of photometer to 620 nm and measure the O.D.

Reagent	*Blank*	*Standard*	*Control*	*Test*
Distilled water	1ml	—	—	—
Glucose std.	—	1ml	—	—
Filtrate of 'C'	—	—	1ml	
Filtrate of 'S'	—	—	—	1ml
Alk. Cu reagent	1ml	1ml	1ml	1ml
	Boiling water bath for 10 min.			
Phosphomolybdate	2 ml	2 ml	2 ml	2 ml

Table showing Folin-Wu procedure

O- TOLUIDINE METHOD

Principle

The aldehyde group of glucose condenses with O- toluidine in glacial acetic acid, which on heating gives an emerald-blue green colour which is measured photometrically. The intensity of colour is directly proportional to the glucose concentration.

Reagents

1. O-toluidine reagent
   Orthotoluidine 60 ml
   Thiourea 1.5 gm
   Glacial acetic acid 1000 ml
2. Glucose stock standard (200 mg/dl in 0.2% benzoic acid solution)
3. Glucose working solution.

Procedure

1. Take three test tubes and label them as blank standard and test.
2. Transfer 1ml distilled water, 1ml working standard, and 1ml test serum to respective tube.
3. To each tube, add 0.9 ml of distilled water and 7 ml of O-toluidine reagent. Cover the tubes with loose cap or aluminium foil.
4. Place in a boiling water bath for 10 min. work inside the hood as it gives strong smell, which is injurious.
5. Cool the tubes for 2-3 min under running tap water.
6. Read the absorbance within 30 min of cooling at 630 nm.

Reagent	*Blank*	*Standard*	*Test*
Distilled water	1 ml	—	—
Glucose std.	—	1 ml	—
Test serum	—	—	1 ml
Distilled water	0.9 ml	0.9 ml	0.9 ml
O-toluidine reagent	7 ml	7 ml	7 ml

Table showing O- Toluidine procedure

GLUCOSE OXIDASE METHOD

Principle

Glucose oxidase (GOD) oxidizes glucose to gluconic acid. Hydrogen peroxide is produced in this reaction. In presence of peroxide hydrogen peroxidase (POD) reacts with 4-aminoantipyrine and phenol to form red coloured quinoneimine dye. The intensity of colour is directly proportional to the glucose concentration. This method is highly specific for glucose and does not involve any other sugar.

$$\text{Glucose} + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{POD}} \text{Quinoneimine dye}$$

Reagents

1. Glucose oxidase reagent
2. Phenol solution
3. Glucose stock standard (200 mg/dl in 0.2% benzoic acid solution)
4. Glucose working solution.

Procedure

1. Take three test tubes and label them as blank standard and test.
2. Add 2 ml of glucose oxidase reagent into the three test tubes.
3. Add 0.5 ml of distilled water; 0.5ml of ten times diluted serum, 0.5ml of working standard in blank, test and serum respectively.
4. Add 2 ml of phenol reagent into the three test tubes.
5. Shake well and allow it to stand for 30 min. at room temperature or 15 min. at 37°C.
6. Read the absorbance at 515 nm.

Reagent	*Blank*	*Standard*	*Test*
Glucose oxidase	2 ml	2 ml	2 ml
Glucose std.	—	0.5 ml	—
Diluted test serum	—	—	0.5 ml
Distilled water	0.5 ml	—	—
Phenol reagent	2 ml	2 ml	2 ml

Table showing glucose oxidase procedure

Calculation

$$\text{Glucose mg/100 ml} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times \text{Conc. of Std.}$$

Normal Values

- Fasting 65 — 110 mg/dl
- Post meal 120 —140 mg/dl
- Random 70 — 140 mg/dl

Clinical Significance

Blood glucose level is mainly determined to diagnose diabetes mellitus. It provides the valuable information about the course, severity and therapeutic control of diabetes mellitus.

With the FPG test, a fasting blood glucose level between 100 and 125 mg/dl signals pre-diabetes. A person with a fasting blood glucose level of 126 mg/dl or higher has diabetes**.** The early symptoms of untreated diabetes mellitus are related to the elevated blood glucose levels. Excess glucose in the blood ultimately results in high levels of glucose being present in the urine (glucosuria). This increases the urine output, which leads to dehydration and increased thirst. Other symptoms include extreme tiredness, weight loss, blurred vision, itchy skin and repeated minor infections such as thrush and boils.

Beside the diabetes, blood sugar level is also increased in —

With increased circulatory epinephrine, pancreatitis, some CNS lesions, or effects of drugs like alcohol, phenytoin, etc

Decreases in—Extrapancreatic tumors, hepatic disease, endocrine disorders, pediatric abnormalities, enzyme diseases and malnutrition.

ADVANCES IN MONITORING DIABETES

1. Hb A1c Test
2. Glucometer

HbA1c Test

(Also called as Glycosylated hemoglobin; Hemoglobin-glycosylated; A1c; GHb; Glycohemoglobin; Diabetic control index)

Definition

HbA1c is a test that measures the amount of glycosylated hemoglobin in the blood. The test gives a good estimate of how well diabetes is being managed over time.

Normally, only a small percentage of the hemoglobin (Hb) molecules in red blood cells become glycosylated (that is, chemically linked to glucose). The percent of glycosylation increases over time, and is higher if there is more glucose in the blood. Therefore, older red blood cells will have a greater percent of glycosylated hemoglobin, and diabetics whose blood glucose has been too high will have a greater percent of glycosylated hemoglobin.

This test measures blood sugar control over an extended period in people with diabetes. In general, the higher the HbA1c value, the higher the risk that a person will develop problems such as eye disease, kidney disease, nerve damage, heart disease, and stroke. This is especially true if the HbA1c remains elevated for more than one occasion.

Normal Values

HbA1c is normal if it is 5 percent or less. The test can show that the blood glucose levels have not been well-regulated over a period of weeks to months. If the HbA1c value is above 7%, it means diabetes is poorly controlled. High values indicates greater risk of diabetic complications. This test is recommended usually every 3 or 6 months.

Glucometer

A glucose meter (or glucometer) is a medical device for determining the approximate amount of glucose in a drop of blood obtained by pricking the skin with a lancet. Glucose meters are portable and designed for use by laypersons, including those with diabetes.

The glucose meter is a key element of *home blood glucose monitoring* by people with diabetes mellitus or with proneness to hypoglycemia.

There are now dozens of models of glucose meters are available. Typical features common to most are:

- The average size is now approximately the size of the palm of the hand. They are battery powered.
- A consumable element containing chemicals, which react with glucose in the drop of blood, is used for each measurement. For most models this element is a plastic *test strip* with a small spot impregnated with glucose oxidase and other components. Each strip can only be used once and is then discarded.
- The glucose value in mg/dl or mmol/l displayed in a small window.
- Glucose levels in plasma are generally 10-15% higher than glucose measurements in whole blood (and even more after eating). This is important because home blood glucose meters measure the glucose in whole blood while most lab tests measure the glucose in plasma.
- Current "count times" range from 5 to 60 seconds for different models.
- The size of the drop of blood needed by different models currently varies from 0.3 to 10 µl.
- All meters now include a *clock*, which must be set for date and time, and a *memory* for past test results. The memory is an important aspect of diabetes care, as it enables the person with diabetes to keep a record of management and look for trends and patterns in blood glucose levels over days. Most memory chips can display an average of recent glucose readings.
- Many meters have now had more sophisticated data handing capabilities. Many can be downloaded by a cable or infrared to a computer which has software to display the test results in a variety of formats. Some meters allow entry of additional data throughout the day, such as insulin dose, amounts of carbohydrates eaten, or exercise.
- A number of meters have been combined with other devices, such as insulin injection devices, PDAs. A radio link to an insulin pump allows automatic transfer of glucose readings to a calculator that

assists the wearer in deciding on an appropriate insulin dose. One model also measures beta-hydroxybutarate in the blood to detect ketoacidosis.

- Special glucose meters for multi-patient hospital use are now used. These provide more elaborate quality control records, and the data handling capabilities are designed to transfer glucoses into electronic medical records and the laboratory computer systems for billing purposes.

The cost of daily testing is one of the most expensive aspects of diabetes care. In 2006, the consumer cost of each glucose strip ranges from about Rs. 17 to 50.

Accuracy of glucose meters is a common topic of clinical concern. Nearly all of the meters have similar accuracy (±10-15%) when used optimally. However, a variety of factors can affect the accuracy of a test. Factors affecting accuracy of various meters have included calibration of meter, ambient temperature, pressure use to wipe off strip, size of blood sample, high levels of certain drugs in blood, hematocrit, dirt on meter, humidity, and aging of test strips.

GLUCOSE TOLERANCE TEST (GTT)

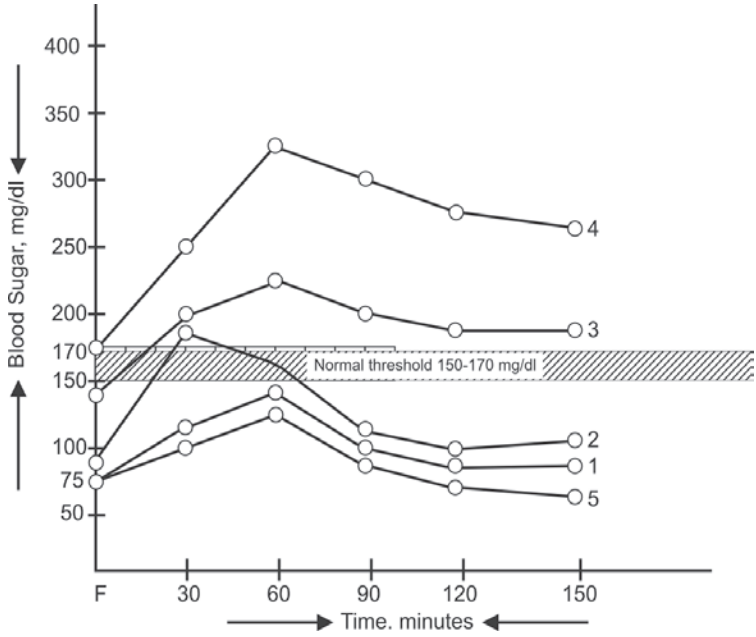
Glucose tolerance test means ability of the body to utilize glucose in blood circulation. Glucose tolerance decreases in certain diseases like diabetes mellitus and endocrine disorders.

Blood glucose in case of normal individual remains fairly constant throughout the day. Except, after meals, there is temporary rise, which comes to normal within 2-3 hr. of meal. In decreased tolerance level however, the blood sugar level does not return to normal within 2-3 hrs. of meal. Glucose tolerance test determines the degree and duration of hyperglycemia after an oral intake of known quantity of glucose.

The patient is prepared for the test by being kept on a diet containing 300 g of carbohydrate a day for three days before the test. Patient should come to lab in the morning after overnight fasting of 12-16 hr. Patient is not allowed to have tea/coffee/medicine/smoke/tobacco chew.

Procedure

1. First, collect the fasting urine and blood sample. If glucose is present in urine, do not perform GTT, instead take a post meal sample.
2. If glucose is absent, give patient 75 to 100 gm glucose (1.50 gm/ kg body weight) dissolved in water. Note the time.
3. Thereafter the blood and urine samples are collected for every 30 min for two and half hr.
4. Determine blood and urine sugar.
5. Prepare a Glucose tolerance curve by plotting time on X- axis and glucose values on Y-axis (Fig. 15.2).



(1) Normal (2) Lag type (3) Mild diabetic (4) Severe diabetic

Fig. 15.2: GTT curves

INTERPRETATION

1. *Glucose tolerance curve No.1:* This is a normal type of curve. It shows fasting glucose within normal limits. Maximum blood glucose level is reached either half or one hour after taking glucose. Within 2 hrs it comes rapidly to normal limits. Glucose should not present in any of urine sample.
2. *Glucose tolerance curve No.2:* This type of curve is termed as Lag curve. Here the peak of blood glucose level may be higher than normal but the 2 hr value is within normal limits or often low. The increase in blood glucose level is due to delay in insulin mechanism coming into action. If the blood glucose level at peak of the curve is above the renal threshold level, glucose appears in next urine sample. Such a curve is seen in—— Normal individuals, after gastrectomy and in severe liver disease.
3. *Glucose tolerance curve No.3 and 4:* This is present when ability of body to utilize glucose decreases. The rise in blood glucose is greater than in normal persons and the return of blood glucose to normal fasting level is delayed. GTC No. 3 indicates mild diabetes and GTC No. 4 indicates severe diabetes. This type of curve may be seen in hyperactivity of hormones, very severe liver disease and severe infection.
4. *Glucose tolerance curve No.5:* This curve indicates increased glucose tolerance i.e., ability of the body to utilize more glucose. The fasting blood sugar may be below usual limits and only a small rise in blood glucose is observed. This type of curve may be observed with hypoactivity of endocrine gland, in patients with idiopathic steatorrhea and sprue.

URINE GLUCOSE DETERMINATION

When blood sugar level exceeds 170 mg/ dl, glucose appears in urine. This condition is called as glycosuria. Increased concentration of glucose in urine indicate proportionate increase in blood sugar level.

Qualitative Test for Sugar Determination Test for Glucose (or Reducing Substances Like Fructose, Lactose, Galactose, Pentose)

- *Benedict's test (Qualitative):* Take 5 ml of Benedicts qualitative reagent in test tube and add 0.5 ml of urine. Boil the content of

tube. Let it stand on the rack for 5 to 10 min. The appearance of a yellow or red deposits indicates the presence of reducing substances i.e. sugar. Cupric sulphate is reduced to cuprous oxide by boiling with reducing agents.

Report: A slight green colour, light turbidity or a bluish white ppt or no change is reported as negative. A greenish colour with a little yellow deposit is reported as a trace (+), green yellowish (++), orange (+++) and brick red (++++).

Nowadays, paper strips are available commercially which are dipped in urine as directed and the colour produced is matched against the colour chart supplied.

Quantitative Test for Sugar Determination

Reducing sugars (glucose) reacts with Benedict's quantitative reagent. Here glucose reduces cupric ions to cuprous ions which reacts with potassium thionate in the reagent to form white ppt. This is very sharp reaction and easy to detect.

Procedure

1. Pipette 5 ml of the Benedict's quantitative reagent in a porcelain dish.
2. Add 2-3 gm anhydrous sodium carbonate and mix well.
3. Heat the mixture to the boiling point.
4. Add urine dropwise with constant stirring with glass rod till the blue colour of the reagent disappears and white ppt. is formed.
5. Note the titration reading.

Calculations

$$\text{Urinary glucose mg/ dl} = \frac{10 \times 10}{\text{Titration reading (ml)}}$$

Normal Value

0 to 0.3 gm /24 hrs.

Causes of Glycosuria

Diabetes mellitus, non-diabetic glycosuria includes emotional disturbances, hyperthyroidism, pregnancy, after ingestion of considerable carbohydrates, either anaesthesia, in some infections, like pneumococcal pneumonia, etc.

16 Liver Function Test

The liver is the largest organ in the human body, located in the right upper quadrant of the abdomen. The liver is of vital importance in intermediary metabolism, in the hematopoiesis and in the elimination of toxic substances.

Liver function test includes:

a. Serum bilirubin
b. SGPT
c. SGOT
d. Serum protein
e. Alkaline phosphatase
f. Urine — bile salts, bile pigments and urobilinogen

The interpretation of liver function test plays a key role to diagnose jaundice, parenchymal diseases (like hepatitis, cirrhosis, fatty liver and infiltration) chronic hepatitis, altered drug metabolism, endocrine abnormality, nutritional and metabolic abnormality, etc.

Serum Bilirubin

Bilirubin originates from the break down of haemoglobin. It is a waste product and the body eliminates this compound through the bile (from liver) into the intestine and ultimately through the stool. Only a small fraction of bilirubin metabolite is recycled through the body and a part of it is excreted through the urine.

Serum Bilirubin can be present in two forms:

1. *Conjugate bilirubin:* It is conjugated with glucuronic acid to form bilirubin glucuronide. It is water-soluble. It reacts directly in aqueous solution without alcohol. Therefore it is also called as direct bilirubin.

2. *Unconjugate bilirubin:* It is a free bilirubin formed from protoporphyrin component of heme. It is insoluble in water and carried away to liver by serum albumin. It reacts indirectly with presence of alcohol. Therefore it is also called as indirect bilirubin. *Specimen:* Serum is preferred for determination of bilirubin. It should not be haemolysed. Protect it from light. Plasma can be used with anticoagulant like heparin. Generally fasting samples are preferred.

METHODS

I. Malloy and Evelyn method
II. Jendrassik-Grof method

MALLOY AND EVELYN METHOD

Principle

Bilirubin couples with diazotized sulphanilic acid to form a purple coloured azobilirubin complex . Direct bilirubin reacts with the diazo reagent in aqueous solution to form a coloured diazo compound within 1 min. the indirect bilirubin is diazotized only in the presence of methanol. The subsequent addition of methanol accelerates the reaction of indirect bilirubin . The value of total bilirubin is obtained after letting the specimen stand for 30 min. The absorbance values of coloured solution are taken at 540 nm.

Reagents

1. Diazo reagent A and B
2. Methanol
3. Conc. HCl
4. Working bilirubin std. (10 mg %)
5. Diazo blank reagent (1.5 ml Conc. HCl diluted to make 100 ml with distilled water)

Procedure

1. Prepare Diazo mixture by adding 5 ml Diazo A and 0.15 ml of Diazo B.

2. Take four test tubes. Label them as, TT (total test),TB (total blank), DT (direct test), and DB (direct blank)
3. Add the reagents as ——
   i. 0.1ml serum and 0.9 ml distilled water in each tube.
   ii. 0.25ml Diazo blank in TB and DB
   iii. 0.25 ml Diazo reagent in TT and TB
   iv. 1.25 ml distilled water to DT and DB
   v. 1.25 ml methanol to TT and TB.
4. Mix well and read the OD of DT and DB after one min. against distilled water at 540 nm.
5. Mix well and keep the tubes TT and TB in dark at room temperature for 30 min. and read the OD against distilled water at 540 nm.
6. Read OD of bilirubin std.(reagent 4) against distilled water.

Reagent	*TT*	*TB*	*DT*	*DB*
Serum	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Distilled water	0.9 ml	0.9 ml	0.9 ml	0.9 ml
Diazo blank	—	0.25 ml	—	0.25 ml
Diazo reagent	0.25 ml	—	0.25 ml	—
Distilled water	—	—	1.25 ml	1.25 ml
Methanol	1.25 ml	1.25 ml	—	—

Table showing Malloy and Evelyn method

Calculation

$$\text{Total bilirubin (A)} = \frac{\text{O.D. of TT} - \text{O.D. of TB}}{\text{O.D. of Std.}} \times 10$$

$$\text{Direct bilirubin (B)} = \frac{\text{O.D. of DT} - \text{O.D. of DB}}{\text{O.D. of Std.}} \times 10$$

Indirect bilirubin = A – B.

JENDRASSIK-GROF METHOD

Principle

Bilirubin reacts with diazotized sulphonilic acid in presence of a strong alkaline tartrate solution gives blue azobilirubin solution. This is a

reaction of direct bilirubin. Indirect bilirubin reacts with diazo reagent in presence of the accelerator caffeine benzoate. This reaction represents total bilirubin.

Reagents

1. 0.05 N HCl
2. Caffeine benzoate reagent
3. Diazo reagent
4. Ascorbic acid solution
5. Alkaline tartrate
6. Normal saline

Procedure

Part I. Direct Bilirubin

1. Dilute the specimen by mixing 1ml of specimen with 4ml of saline.
2. Take two test tubes. Label them as, DT (direct test), and DB (direct blank).
3. To the tube DT, add 0.5ml of diazo reagent and exactly after 1 min. add 1.5 ml alkaline tartrate.
4. Add 1ml of diluted serum and 2 ml of 0.05 N HCl in both the tubes. Mix and after 10 min. read the absorbance.
6. To DB, add 0.5 ml diazo A, 0.5 ml ascorbic acid and 1.5 ml alkaline tartrate. Mix and take the reading without waiting.

Reagent	*DT*	*DB*
Diazo reagent	0.5 ml	0.5 ml
Ascorbic acid	—	0.5 ml
Alkaline tartrate	1.5 ml	1.5 ml
Diluted serum	1 ml	—
0.05 N HCl	2 ml	—

Part II. Total Bilirubin

1. Take two test tubes. Label them as, TT (Total test), and TB (total blank)
2. Place 1ml of diluted serum and 2.1 ml Caffeine benzoate reagent in both the tubes.

3. Add 0.5 ml of Diazo reagent in test.
4. After 10 min. add 1.5 ml of alkaline tartrate and take the absorbance after 10 min.
5. To the tube TB, add 0.5 ml Diazo A and then 1.5 ml alkaline tartrate.

Reagent	*TT*	*TB*
Dil. Serum	1 ml	1 ml
Caffeine benzoate	2.1ml	2.1 ml
Diazo reagent	0.5ml	—
Alkaline tartrate	1.5 ml	—
Diazo reagent	—	0.5 ml
Alkaline tartrate	—	1.5 ml

Calculation

$$\text{Total bilirubin (A)} = \frac{\text{OD of TT}}{\text{OD of Std.}} \times \%\ \text{of Std.}$$

$$\text{Direct bilirubin (B)} = \frac{\text{OD of DT}}{\text{OD of Std.}} \times \%\ \text{of Std.}$$

Indirect bilirubin = A – B

Normal values:

Total bilirubin	Age	Value
	Newborn	up to 5.8 mg/dl
	1-2 days	up to 8.2 mg/dl
	3-5 days	up to 11.7 mg/dl
	Above one month to adult	up to 1.0 mg/dl
Direct bilirubin		0.0 to 0.2 mg /dl
Indirect bilirubin		0.4 to 0.8 mg /dl

Clinical Significance

Determination of serum bilirubin is important in diagnosis of diseases of hepatobiliary system and pancreas and other causes of jaundice.

Jaundice becomes apparent clinically when serum bilirubin level goes more than 2.5 mg/dl.

Increased direct bilirubin is seen in Hepatic cellular damage, liver diseases related to viral, toxic, alcohol or drugs, Biliary duct obstruction, Infiltrations, Space occupying lesions, live metastatic tumor, etc.

Increased indirect bilirubin is seen in hemolytic diseases, ineffective erythropoiesis, Blood transfusions, Haematomas, Hereditary disorders (e.g. Gilbert's disease)

TRANSAMINASES

Transamination is a process in which an amino group is transferred from an amino acid to an alpha keto acid. It is an important step in amino acid metabolism. The enzymes responsible for transamination are called Transaminases. Two diagnostically useful transaminases are Glutamate oxaloacetate transaminase or GOT (also called as asparate aminotransferase or AST) and Glutamate pyruvate transaminase or GPT (also called as alanine aminotransferase or ALT).

L-asparate + Oxoglutarate $\xrightarrow{\text{GOT/AST}}$ Oxaloacetate + Glutamate

L-alanine + Oxoglutarate $\xrightarrow{\text{GPT/ALT}}$ Pyruvate + Glutamate

SGPT (ALT)

Method

2- 4- DNPH Method

Specimen

Serum is required for the test. Haemolysis should be avoided.

Principle

GOT catalyses following reaction—

L-alanine + Oxoglutarate $\xrightarrow{\text{GPT / ALT}}$ Pyruvate + Glutamate

Pyruvate so formed is coupled with 2,4- Dinitrophenyl hydrazine (2,4-DNPH) to corresponding hydrazone, which gives brown colour in alkaline medium. This can be measured colorimetrically.

Reagents

1. Buffered alanine α-KG substrate at pH 7.4
2. DNPH colour reagent
3. 4N NaOH
4. Working pyruvate std. 2 mm

Procedure

Dilute 1 ml of 4N NaOH to 10 ml purified water. This makes the working solution.

Part I. Preparation of Standard Curve

Standardization is done against the standard Karmen unit Assay and this is extrapolated to different amounts of pyruvate. The standard graph of enzyme activity (in units / ml) on X-axis Vs O.D. on Y-axis is not a linear one. This shows that O.D. increases with increase in enzyme activity at a decreasing rate.

Take five test tubes, mark them as — 1, 2, 3, 4, and 5 for corresponding enzyme activity —0, 28, 57, 97 and 150. Add the reagents as follows:

Tube no.	*1*	*2*	*3*	*4*	*5*
Enzyme activity units /ml	0	28	57	97	150
Buffered alanine at pH 7.4	0.5 ml	0.45 ml	0.4 ml	0.35 ml	0.3 ml
Working pyruvate std. 2 mm	—	0.05 ml	0.1 ml	0.15 ml	0.2 ml
Purified water	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DNPH colour reagent	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Table showing SGPT procedure

Mix well and allow it to stand at RT for 20 min. Add 5 ml of diluted NaOH to each tube. Mix well by inversion. Allow it to stand at RT for 10 min measure the OD of all the five tubes against purified water on a colorimeter at 505 nm.

Part II. Preparation of Test

For test, take 0.25 ml of buffered alanine at pH 7.4 in a test tube. Incubate at 37°C for 5 min. Add 0.05 ml serum to the test tube and again incubate at 37°C for 30 min. Now add 0.25 ml DNPH colour reagent. Mix well by inversion. Allow to stand at RT for 20 min. At last add 2.5 ml diluted NaOH. Mix well and allow it to stand at RT for 10 min. Now measure the OD against purified water on a colorimeter at 505 nm.

Calculations

Mark the OD of test on Y- axis of std. curve and extrapolate it to the corresponding enzyme activity on X- axis.
Normal Values:

Age	*SGPT level*	
1-3 yr	5-45 U/L	
4-6 yr	10-25 U/L	
7-9 yr	10-35 U/L	
	Male	*Female*
10-11 yr	10-35 U/L	10-30 U/L
12-13 yr	10-55 U/L	10-30 U/L
14-15 yr	10-45 U/L	5-30 U/L
16-19 yr	10-40 U/L	5-35 U/L

Determination of SGPT level is important in differential diagnosis of diseases of hepatobiliary system and pancreas.

Increased SGPT levels are found in severe preeclampsia, rapidly progressing acute lymphoblastic leukemia, obesity, etc.

Decreased SGPT levels are found in genitourinary infection malignancy, malnutrition, pregnancy, alcoholic liver disease, etc.

SGOT (AST)

Method

2- 4- DNPH Method

Specimen

Serum is required for the test. Haemolysis should be avoided.

Principle

GOT catalyses following reaction—

$$\text{L-asparate} + \text{Oxglutarate} \xrightarrow{\text{GOT / AST}} \text{Oxoacete} + \text{Glutamate}$$

Oxalate so formed is coupled with 2,4- Dinitrophenyl hydrazine (2,4-DNPH) to corresponding hydrazone, which gives brown colour in alkaline medium. This can be measured colorimetrically.

Reagents

1. Buffered aspirate α-KG substrate at pH 7.4
2. DNPH colour reagent
3. 4N NaOH
4. Working oxalate std. 2 mm

Procedure

Dilute 1 ml of 4N NaOH to 10 ml purified water. This makes the working solution.

Part I. Preparation of Standard Curve

Standardization is done against the standard Karmen unit Assay and this is extrapolated to different amounts of oxalate. The standard graph of enzyme activity (in units/ml) on X-axis Vs OD on Y-axis is not a linear one. This shows that OD increases with increase in enzyme activity at a decreasing rate.

Take five test tubes, mark them as — 1, 2, 3, 4, and 5 for corresponding enzyme activity — 0, 24, 61, 114 and 190. Add the reagents as follows:

Tube no.	*1*	*2*	*3*	*4*	*5*
Enzyme activity units /ml	0	24	61	114	190
Buffered asparate at pH 7.4	0.5 ml	0.45 ml	0.4 ml	0.35 ml	0.3 ml
Working oxalate std. 2mm	—	0.05 ml	0.1 ml	0.15 ml	0.2 ml
Purified water	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DNPH colour reagent	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Table showing SGOT procedure

Mix well and allow it to stand at RT for 20 min. Add 5ml of diluted NaOH to each tube. Mix well by inversion. Allow it to stand at RT for 10 min. measure the OD of all the five tubes against purified water on a colorimeter at 505 nm.

Part II. Preparation of Test

For of all test, take 0.25 ml of buffered asparate at pH 7.4 in a test tube. Incubate at 37°C for 5 min. Add 0.05 ml serum to the test tube and again incubate at 37°C for 60 min. Now add 0.25 ml DNPH colour reagent. Mix well by inversion. Allow to stand at RT for 25 min. At last add 2.5 ml diluted NaOH. Mix well and allow it to stand at RT for 10 min. Now measure the OD against purified water on a colorimeter at 505 nm.

Calculations

Mark the OD of test on Y- axis of std. curve and extrapolate it to the corresponding enzyme activity on X- axis.
Normal Values:

Age	*SGOT level*
1-3 yr	20-60 U/L
4-6 yr	15-50 U/L
7-9 yr	15-40 U/L
10-11 yr	10-60 U/L

	Males	*Females*
12-15 yr	15-40 U/L	10-30 U/L
16-19 yr	15-45 U/L	5-30 U/L

Determination of SGOT level is important in differential diagnosis of diseases of hepatobiliary system and pancreas.

Increased SGOT levels are found in liver diseases like cirrhosis, hepatic ischema biliary obstruction, granulomas,etc. SGOT is also increased in — cerebral infarction, burns, intestinal injury, acute pancreatitis, etc. Marked increase, i.e. above 3000 U/L is found in-acute hypotension, toxic liver injury, liver trauma, viral hepatitis, etc.

Decreased SGOT levels are found in Azotemia, chronic renal dialysis, malnutrition, pregnancy, alcoholic liver disease, etc.

The normal ratio of SGOT/ SGPT is, 0.7 to 1.4. it is found increased in –Drug hepatotoxicity (> 2.0) , Alcoholic hepatitis (≤ 6.0), Cirrhosis (1.4 – 2.0) intrahepatic cholestasis (> 1.5) and chronic hepatitis.

SERUM PROTEINS

Serum proteins constitute of albumin and globulin. Its detection is useful in diagnosis of liver diseases.

Method

Biuret method.

Specimen

Serum. Haemolysis is strictly avoided.

Principle

Proteins react with cupric ions in alkaline medium to form a violet coloured complex. The intensity of the colour produced is directly proportional to proteins present in the specimen and can be measured at 530 nm.

Reagents

Biuret Reagent
Protein std. (6g/dl)

Procedure

Take three test tubes. Mark them as T, S and B add 5 ml of Biuret reagent in each tube. Add 0.05 ml of serum, 0.05 ml of protein std.

and 0.05 ml of distilled water in test, std. and blank respectively. Mix thoroughly and keep at room temperature for exactly 10 min. Measure the intensities of test and std. against blank at 530 nm

Reagent	*T*	*S*	*B*
Buriet reagent	5 ml	5 ml	5 ml
Serum	0.05	—	—
Protein std.	—	0.05	
Distilled water	—	—	0.05

Calculations

$$\text{Serum proteins} = \frac{\text{OD of T}}{\text{OD of S}} \times 6$$

Normal values

Age	Total proteins (gm/dl)
< 5 days	5.4 - 7.0
1 - 3 years	5.9 - 7.0
4 - 6 years	5.9 - 7.8
7 - 9 years	6.2 - 8.1
10 - 19 years	6.3 - 8.6

Clinical Significance

Determination of serum total proteins is useful in screening for nutritional deficiencies and gammopathies.

It is increased in multiple myeloma, Hypergammaglobulinemias, hypovolemic states. It is often found lower than limits in nutritional deficiency like Kwashiorkor and Marasmus. Decreased protein synthesis like in case of severe liver disease, increased protein loss like in severe skin disease, GI disease, Renal disease and blood loss. Increased catabolism like in case of fever or inflammation, malignancy etc.

SERUM ALBUMIN

Albumin is one of the important proteins, synthesized in liver.

Method

Bromocresol green method

Principle

Albumin binds specifically with Bromocresol green at pH 4.1 to form green coloured complex. Intensity of the colour is directly proportional to the amount of Albumin present in the sample. The colour is measured at 640 nm.

Specimen

Serum

Reagents

Albumin reagent
All standard (4.0 g/dl)

Procedure

Take three test tubes, Mark them as Test, Std. and Blank. Add 5ml Albumin reagent, in each tube; add 0.05ml serum, 0.05ml Albumin Std. and 0.05 ml distilled water to test, Std. and Blank respectively.

Reagent	*T*	*S*	*B*
Albumin reagent	5 ml	5 ml	5 ml
Serum	0.05	—	—
Albumin Std.	—	0.05	—
Distilled water	—	—	0.05

Mix thoroughly and keep at room temperature for exactly 10 minutes. Measure the intensity of the test and Std. against blank at 640 nm

Calculation

$$\text{Serum Albumin} = \frac{\text{OD of T}}{\text{OD of S}} \times 4$$

Normal values –

Age	Values
< 5 days	2.6 - 3.6
1 - 3 years	3.4 - 4.2
4 - 6 years	3.5 - 5.2
7 - 9 years	3.7 - 5.6

Clinical Significance

Determination of serum albumin is important in disorders of protein metabolism.

Albumin levels are high in dehydration and intravenous albumin infusions.

Albumin is found to be decreased in – malnutrition, decreased absorption, liver diseases, chronic infection, hyperthyroidism, pregnancy, burns, hemorrhage etc.

SERUM GLOBULIN

Serum Globulin can be obtained when the values of total protein and serum albumin are known.

Total Protein = Serum Albumin + Serum Globulin

$\therefore$ Serum Globulin = Total protein – Serum Albumin

Normal Values

Age	Values
< 1 year	0.4 - 3.7
1 - 3 years	1.6 - 3.5
4 - 9 years	1.9 - 3.4
10 - 49 years	1.9 - 3.5

A/G Ratio:

$$\text{A/G Ratio} = \frac{\text{Serum Albumin}}{\text{Serum Globulin}}$$

Normal Value – 1.2 : 1 to 2 : 1

Clinical Significance

Calculation of A/G ratio is also helpful in diagnostic interpretation for liver disease. An alteration in the A/G ratio and reversal may occur

due to the reduction in albumin and or elevation of globulin. The ratio is reduced and often reversed in cirrhosis with jaundice. However the ratio may be increased in some cases of xanthomatosis or billary cirrhosis.

ALKALINE PHOSPHATASE

Alkaline Phosphatase is present in most tissues but is present in high concentration in liver, bones, intestines, spleen, placenta and kidney. It is involved in transport of phosphate across cell membrane. It has hydrolytic and phosphate transferase activity.

Method

Kind and King's method.

Specimen

Serum is preferred, but heparinized plasma can also be used. Other anticoagulants inhibit the enzyme activity. Overnight fasting serum is preferred; store the serum in refrigerator if immediate analysis is not possible.

Principle

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10. Phenol so formed reacts in alkaline medium with 4-Aminoantipyrine in presence of the oxidizing agent potassium ferrocyanide and forms an orange-red coloured complex which can be measured colorimetrically. The colour intensity is proportional to enzyme activity.

$$\text{Phenyl phosphate} \xrightarrow{\text{Alkaline Phosphatase}} \text{Phenol} + \text{Phosphate}$$

$$\text{Phenol} + \text{4 – amino Antipyrine} \xrightarrow[OH^-]{\text{Pot. ferricyanide}} \text{Orange red coloured complex}$$

Reagents- Substrate – Disodium Phenyl Phosphate
Bufffer – $NaHCO_3$ + Na_2CO_3 (pH10)
Phenol Std. – 0.01mg/ml
NaOH
4- Amino Antipyrine
Potassium ferricyanide oxidizing agent.

Procedure

Take four test tubes. Mark them as –Test, Control, Std and Blank. Add the reagents according to following table.

Reagents	*T*	*C*	*S*	*B*
Buffer Substrate	1 ml	1 ml	—	—
Buffer	1 ml	1 ml	1.1 ml	1.1ml
Phenol Std.	—	—	1 ml	—
Distilled water	—	—	—	1 ml
Serum	1 ml	—	—	—
Incubate at 37°C for 15 minute				
NaOH	0.8 ml	0.8 ml	0.8 ml	0.8 ml
Serum	—	0.1 ml	—	—
4- amino antipyrine	1 ml	0.8 ml	1 ml	1 ml
Pot. ferricyanide	1 ml	0.8 ml	1 ml	1 ml

Mix after addition of each reagent and measure the OD of T,C,S and B against D/W at 540 nm after 10 minute.

Calculations

$$\text{Serum ALP In KA units} \quad \frac{\text{OD of Test} - \text{OD Control}}{\text{OD std.} - \text{OD Blank}} \times 0.10$$

Normal Values
1 - 3 years 145-320 U/L
4 - 6 years 150-380 U/L
7 - 9 years 175-420 U/L

	Males	*Females*
10-11 years	135-530 U/L	130-560 U/L
12-13 years	200-495 U/L	105-420 U/L
14-15 years	130-525 U/L	70-230 U/L
16-19 years	65-260 U/L	50-130 U/L

Clinical Significance

Determination of alkaline phosphatase is important in diagnosis of causes and monitoring of course of cholestasis (e.g. Neoplasm, drugs). It is also helpful in diagnosis of various bone disorders (e.g. Paget's disease)

ALP is increased in - Bone disorders like osteomalacia, Hodgkin's disease, increased deposition of calcium etc. Liver diseases like liver infiltrates, nodules in liver, hepatic congestion due to heart disease 44% of diabetic patients have 40% increase of ALP. 15 to 20 times increase in ALP is found in primary cirrhosis and liver cancer.

ALP is decreased in – Excess vitamin D ingestion, celiac disease, malnutrition, scurvy, zinc deficiency, Mg. deficiency, and hypothyroidism. In one-third patients of pernicious anemia, ALP level is decreased.

URINE ANALYSIS – BILE SALT, BILE PIGMENT AND UROBILINOGEN

INTRODUCTION

Bile is a yellow-green fluid that is made by the liver. It is stored in the gallbladder and passes through the common bile duct into the duodenum where it helps to digest fat. The principal components of bile are cholesterol, bile salts, and the pigment bilirubin.

Conjugated bilirubin is excreted into the biliary canaliculi and then through the bile duct, it passes to the intestine. In large intestine, it is reduced by bacterial action to a group of colourless chromogen including urobilinogen. A small fraction of urobilinogen is absorbed into the portal circulation. Partly it is re-excreted in bile while kidneys excrete the remainder. Most of the urobilinogen is excreted in the feces where it is oxidized by air to the pinkish brown urobilin.

Bile Salts (Hay's test)

Bile salts consist of glycocholic acid and taurocholic acid. They lower the surface tension of the fluid and thus cause sulphur particles to sink.

Take about 3 to 5 inch column of urine in a small beaker or in a test tube. Sprinkle finely powered dry sulfur over the surface from a height of about half-inch. If bile salts are present, the sulfur powder will sink at bottom.

The presence of bile salts indicates obstructive jaundice.

Bile Pigments

It is present in urine in obstructive jaundice and hepatocellular jaundice. The detection test includes:

a. *Fouchet's test:* This is the most sensitive test. If the urine is alkaline or neutral, acidify it with few drops of 2% acetic acid. To about 10 ml of acidic urine add about 5 ml of 10% barium chloride solution. Mix well and filter. To the residue on the filter paper add a drop of Fouchets reagent. A green or blue colour indicates presence of bile pigment's, i.e. biliverdin and bilirubin respectively.
b. *Smith's test:* Take 3 ml of urine in a test tube, over lay with equal amount of diluted tincture iodine (equal volume of distilled water and tincture iodine.) A bright ring develops at the junction of the fluids if bile pigment is present.

Urobilinogen

If the urine sample contains bile pigment it should be removed by addition of 1 part of 10% aqueous solution of calcium chloride to 4 parts of urine and filtering it.

To 10 ml of fresh urine, add 1 ml of Ehrlich Aldehyde Regent. Allow it to stand for 3 min. If red / Cherry colour is obtained, it indicates presence of urobilinogen in urine.

Normal Value < 4.0 mg/dl 24 m.

Clinical Significance

Bile in urine implies increased serum direct bilirubin. It often precedes clinical icterus. May occur without jaundice in early hepatitis, early obstruction or liver metastases.

Complete absence of urine urobilinogen strongly suggests complete bile duct obstruction. Level is normal in incomplete obstruction. Decreased in some phases of hepatic jaundice, Increased in haemolytic jaundice and subsiding hepatitis. Increase may indicate hepatic damage even without clinical jaundice.

For example, some patients with cirrhosis metastatic liver disease, congestive heart failure, presence in viral hepatitis depends on phase of disease.

17 Renal Function Test

The important role of kidney in the body metabolism is formation of urine. The kidney not only excretes waste product from the blood but also preserves essential high threshold substances. It also regulates hydration and electrolytic balance.

Any change in the normal constituent of urine reflects improper functioning of kidneys.

The important blood and urine parameters that are measure of kidney functions are urea, uric acid and creatinine.

SERUM UREA

Urea is the major end product of protein metabolism in human body. Urea is synthesized in the liver by urea cycle and is excreted by the kidney. Urea constitutes the major non-protein nitrogen (NPN) of the blood. It represents 45-50% NPN of the blood. It is also the major NPN substance excreted in the urine.

In some countries blood urea is represented as blood urea nitrogen (BUN).

When urea standard is used, the value comes out is blood urea and if urea nitrogen standard is used the value is in terms of BUN. The values are converted to vice versa by:

BUN = mg % Urea × 0.467

and mg % Urea = BUN × 2.14

Urea can be estimated by following methods:

I. Rate of reaction method – UV Kinetic
II. Berthelot reaction method (end point reaction)
III. Diacetylmonoxime method (DAM method)

RATE OF REACTION METHOD – UV KINETIC

Principle

Urea is hydrolysed to ammonia and carbon dioxide by urease. In presence of ammonia, α- ketoglutarate and glutamate dehydrogenase (GLDH), NADH is reduced to NAD^+.

The rate of decrease in OD is measured at the interval of 30 seconds upto 3 minutes at 340 nm. This is directly proportional to the urea concentration in the specimen

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{- Ketoglutarate} + NADH + H^+ \xrightarrow[\text{GLDH}]{} \text{Glutamate} + NAD^+$$

Specimen–Serum

Reagent

1. Enzyme vials
2. Diluent (at pH8)
3. Urea nitrogen Std.

Procedure

Prepare working reagent by mixing contents of one enzyme vial with 20 ml of the diluent. It is stable at 2-4°C for 20 days. Take 1ml of working reagent in a cuvette, add 0.01ml serum, mix well and note the change in OD/min (Δ AT) after every 30 second up to 3 min. Then take 1 ml of working reagent in a cuvette and add 0.01 ml of urea nitrogen std. (20mg/dl) mix well and note the change in OD/min (Δ AS) after 30 seconds up to 3 min.

$$\text{BUN} = \frac{\Delta\,\text{AT}}{\Delta\,\text{AS}} \times 20$$

BERTHELOT REACTION METHOD (END POINT REACTION)

Principle

The principle is based on the Berthelot reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol in

the presence of hypochlorite to form indophenol, which with alkali gives a blue coloured compound. The intensity of coloured compound can be measured at 546 nm.

Specimen

Serum or heparinized plasma

Reagents

1. Urease/Buffer reagent
2. Phenol reagent
3. Hypochlorite reagent
4. Std. urea nitrogen (20 mg/dl)

Procedure

Take three test tubes. Label them as T, S, and B. Add 0.5 ml of urease reagent in each tube. Add 0.02 ml of serum in test and 0.02 ml of standard in std. Mix well and keep at 37°C for 10 minutes. Add 1 ml of phenol reagent and 1 ml of hypochlorite reagent in each tube. Mix well and keep at 37°C for 10 minutes. Now add 5 ml of distilled water in each tube. Mix thoroughly and read optical densities of test and standard against blank at 546 nm.

Reagent	*Test*	*Std.*	*Blank*
Urease/Buffer reagent	0.5 ml	0.5 ml	0.5 ml
Serum/Plasma	0.02 ml	—	—
Std. Urea nitrogen (20 mg/dl)	—	0.02 ml	—
Mix well and keep at 37 °C for 10 minutes			
Phenol reagent	1 ml	1 ml	1 ml
Hypochlorite reagent	1 ml	1 ml	1 ml
Mix well and keep at 37 °C for 10 minutes			
Distilled water	5 ml	5 ml	5 ml

Calculations

$$\text{Serum urea nitrogen} = \frac{\text{OD of T}}{\text{OD of S}} \times 20$$

DIACETYLMONOXIME (DAM) METHOD

Principle

Urea reacts with diacetylmonoxime in hot medium and in the presence of thiosemicarbazide and ferric ions to form a pink coloured compound. The colour intensity is directly proportional to amount of urea in specimen. It is measured at 520 nm.

Specimen

Serum is preferred, however heparinized plasma or fluoride plasma can be used.

Reagents

1. DAM – TSC Reagent (diacetylmonoxime thiosemicarbazide)
2. Uric Acid Reagent
3. Stock Urea Std. 1g%
4. Working Std. (50 mg%)

Procedure

Take 3 test tubes, mark them as T, S and B, for Test, Std. and Blank respectively. Add the reagents as per following table.

Reagent	*Blank*	*Std.*	*Test*
Distilled water	3.6 ml	3.5 ml	3.5 ml
Blood	—	—	0.1 ml
Working Std. (50 mg %)	—	0.1 ml	—
10 % sodium tungstate	0.2 ml	0.2 ml	0.2 ml
2/3 N sulphuric acid	0.2 ml	0.2 ml	0.2 ml

Mix well and allow to stand for 5 min; centrifuge into 3 test tubes pipette out as follows:

	Blank	Std.	Test
Supernatant	2 ml	2 ml	2 ml
DAM-TSC Reagent	3 ml	3 ml	3 ml
Urea acid Reagent	3 ml	3 ml	3 ml

Mix and plug with cotton and place it in a boiling water bath for exactly 15 min. cool and take the reading at 520 nm.

Calculation

$$\text{Serum Urea Nitrogen} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of Std.} - \text{OD of Blank}} \times 50$$

Blood Urea Nitrogen

Normal Value

Age	Values
1 – 3 years	5 – 17 mg/dl
4 – 13 years	7 – 17 mg/dl
14 – 19 years	8 – 21 mg/dl

Clinical Significance

Determination of BUN is used in diagnosis of renal insufficiency. A BUN of 50 –150 mg/dl implies serious impairment of renal function. Markedly increased BUN (150-250 mg/dl) indicates severely impaired glomerular function. BUN is also increased in Hemorrhage to GI tract, stress, shock, congestive heart failure, acute myocardial infarction, vomiting-diarrhea etc. BUN is found to be lowered in over hydration, severe liver damage, increased utilization of proteins for synthesis, malnutrition, low protein diet, poisoning, hepatitis etc.

SERUM CREATININE

Creatine is a substance that forms when food is converted into energy through a process called metabolism. It is present in muscle, brain and blood in free form as well as in the form of creatine phosphate. Creatinine is largely formed in muscle by irreversible and non-enzymatic removal of water from creatine phosphate. Creatinine (cree-AT-ih-nin) is a compound that is excreted from the body in urine. It is a waste product creatinine levels is measured to monitor kidney function.

It is filtered out of the blood by the kidneys and then passed out of the body in urine. It is filtered at the glomeruli and secreted by the tubules. Creatinine is produced at a steady rate and is affected very little by diet or normal physical activities. If the kidneys are damaged and cannot function normally, the amount of creatinine in the urine decreases while the amount of creatinine in the blood increases. Thus, estimation of creatinine directly reflects the kidney function.

Method

Alkaline-picrate method (Jaffe reaction)

Principle

Creatinine reacts with picric acid in alkaline medium to form a reddish yellow complex. Intensity of which is directly proportional to the concentration of creatinine in the specimen and can be measured at 520 nm.

Specimen

Serum or plasma

Reagents

1. 0.04 M picric acid reagent
2. 10g/dl sodium hydroxide
3. Working creatinine std. 1mg/dl

Prepare alkaline picrate reagent by mixing 4 parts of reagent 1 and 1 part of reagent 2. This working reagent is to be freshly prepared whenever needed.

Procedure

Part I. Preparation of Protein Free Filtrate

Take two test tubes. Mark them as test and std add the reagents as follows:

Reagent	*Test*	*Std.*
Distilled water	3 ml	4 ml
Serum	1ml	—
Standard (1mg/dl)	—	1ml
2/3 N sulfuric acid	0.5ml	—
10g/dl Sodium tungstate	0.5ml	—

Centrifuge the content in the test and get clear filtrate.

Part II. Formation of Colour

Take three test tubes and label it as T, B, and S. Add the reagents as per following table.

Reagent	*Test*	*Std.*	*Blank*
Distilled water	3 ml	3 ml	3 ml
Filtrate	2ml	—	—
Diluted Std.(part I)	—	2ml	—
Alkaline picrate reagent	1ml	1ml	1ml

Mix well and keep at room temperature for 20 min. Read the intensities of test and std. at 520 nm against blank.

Calculations

$$\text{Serum creatinine mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 1$$

Normal Value – S. creatinine :
Fetal – 0.4–0.9
Infant – 0.3–0.7
Male – 0.7–1.4
Female – 0.6–1.1

Serum creatinine determination is useful in the diagnosis of renal insufficiency. Serum creatinine is more specific and sensitive indication of renal disease than BUN.

Serum creatinine levels are high in ingestion of creatinine (roast meat), Muscle disease like – Gigantism and acromegaly, prerenal azotemia and postrenal azotemia. 50 percent loss of renal function is

needed to increase serum creatinine from 1.0 to 2.0 mg/dl. Therefore, it is not sensitive for mild to moderate renal injury. In pregnancy serum creatinine value is found to be decreased.

URIC ACID

Uric acid is the end product of purine metabolism. The first step in the catabolism of purines (adenine and guanine) is their hydrolytic deamination to form xanthine and hypoxanthine. These are then oxidized to uric acid. Uric acid is filtered in the glomeruli and partially reabsorbed by the tubules and then it is excreted in urine.

Serum

Uric Acid-Determined by two methods.

Method

I. Henry-Caraway method
II. Enzymatic method.

I. Henry-Caraway Method

Specimen–Serum

Principle

Uric acid in protein free filtrate reacts with phosphotungstic acid reagent in alkaline medium to form a blue coloured complex. The intensity of colour is measured at 660 nm.

Reagents

1. Deproteinizing reagent
2. Sodium carbonate (10 g/d W/V)
3. Stock phosphotungstic acid reagent
4. Stock uric acid Std (100 mg/dl)

Procedure

Dilute stock phosphotungstic acid to 1:10 and stock uric acid std. to 1:200. In a centrifuge tube, take 5.4 ml of Deproteinizing reagent.

Add 0.6 ml of serum. Mix well and centrifuge at 3000 RPM for 10 minutes. Now take 3 test tubes, labelled them as Test, Std. and Blank. Take 3 ml of filtrate, 3 ml of diluted std. and 3 ml of D/W in T, S, and B tubes respectively. Add 1ml of sodium carbonate and 1ml of diluted phosphotungstic acid in each tube. Mix well and keep in dark for exactly 10 minute. Read OD of test and Std. at 660 nm against blank.

Reagent	*T*	*S*	*B*
Filtrate	3 ml	—	—
Dil. Std	—	3 ml	—
Distilled water	—	—	3 ml
Sodium Carbonate	1 ml	1 ml	1 ml
Dil. Phosphotungstic Acid 1 ml	1 ml	1 ml	1 ml

Calculation

$$\text{Serum Uric Acid} = \frac{\text{OD of T}}{\text{OD of S}} \times 5$$

II. Enzymatic Method

Principle

Enzyme uricase converts uric acid to allantonin and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with phenolic chromogens to form red coloured compound. The intensity of red colour is proportional to the amount of uric acid in the sample.

Reaction

$$\text{Uric Acid} + H_2O \xrightarrow{\text{Uricase}} \text{Allantonin} + H_2O_2$$

$$H_2O_2 + \text{phenolic chromogens} \xrightarrow{\text{peroxidase}} \text{Red Colour Compound}$$

Reagents

Uric Acid reagent
Uric Acid Std. (5 mg/dl)

Specimen

Serum

Procedure

Take 3 test tubes, mark them as T, S and B. Take 0.025ml of serum, 0.025 ml of std. and 0.025 ml of distilled water in tubes T, S and B respectively. To each tube add 1ml of uric acid reagent.

Reagent	*T*	*S*	*B*
Serum	0.025 ml	—	—
Std.	—	0.025 ml	—
Distilled water	—	—	0.025 ml
Uric Acid Reagent	1ml	1ml	1 ml

Allow the tubes to stand for 10 minutes at room temperature. Measure the OD at 590 nm against blank.

Calculation

$$\text{Uric Acid} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 5$$

Normal value:

1 – 3 yr	1.8 – 5 mg/dl
4 – 13 yr	2.2 – 4.7 mg/dl
14 – 19 yr	2 – 5 mg/dl

	Males	Females
10 – 11 yr	2.3 – 5.4 mg/dl	3 – 4.7 mg/dl
12 – 13 yr	2.7 – 6.7 mg/dl	3 – 5.8 mg/dl
14 – 15 yr	2.4 – 7.8 mg/dl	3 – 5.8 mg/dl
16 – 19 yr	4 – 8.6 mg/dl	3 – 5.9 mg/dl

Clinical Significance

Uric acid levels are very liable and show day-to-day and seasonal variation in some person. It is also increased by emotional stress, total fasting, increased body weight, and renal failure. It is found to be increased in gout, leukemia, polycythemia, anaemia, psoriasis, hypo

and hyperparathyroidism. It is increased with high protein, weight reduction diet, alcohol consumption, arteriosclerosis and hypertension. Serum uric acid is increased in 80% patients with elevated serum triglycerides.

Serum uric acid levels are decreased in—Wilson's disease, Fanconi's syndrome, carcinomas, Hodgkin's disease, 5 percent patients of postoperative state (GI surgery, coronary artery bypass) diabetes mellitus. It is also low in healthy adults with isolated defect in tubular transport of uric acid.

URINE UREA

This test is mainly used to assess the protein balance and the amount of dietary protein needed by severely ill patients. Urine urea serves this purpose, as it is a measure of protein breakdown in the body.

Urea is excreted by the kidneys, so excretion of urea can reflect kidney function. The urine urea excretion can be measured to obtain a ratio between the plasma (blood) urea and the urine urea. This ratio (U/P urea) is an indicator of how well the kidneys are able to filter and excrete urea from the bloodstream.

For the determination of urine urea 24 hrs of urine sample diluted to 1:20 is used. Diaectylmonoxime method (serum urea) can be applied.

Urine Urea Nitrogen

$$= \frac{\text{OD of test} - \text{OD of Blank}}{\text{OD of Std.} - \text{OD of Blank}} \times 200 \times \text{dilution of urine}$$

Normal Values

Normal values range from 6 to 17 gm/24 hours or (60 to 90 mg/dl).

Abnormal results are indicated as follows:

Low levels usually indicate:
Malnutrition (inadequate protein in diet), Kidney dysfunction and increased re-absorption.

High levels usually indicate:
Excessive protein intake and increased protein breakdown in the body.

UREA CLEARANCE

Urea is filtered at glomerulus, but it is so diffusible that some is reabsorbed in the tubule. The urea clearance is therefore lower than the glomerular filtration rate (GFR), but can still be a test of some usefulness.

Urea clearance can be defined as the ml of blood (plasma/serum), which contain the urea excreted in a minute by the kidneys.OR The volume of the blood cleared of urea per minute by either renal clearance or hemodialysis.

When there is a good flow of urine (more than 2ml per min.) the proportion of urea, which is reabsorbed, is fairly constant and the clearance is calculated by the usual formula. The value obtained is then called the maximum urea clearance (C_m)

$$C_m = \frac{UV}{P}$$

Where,
V = Urine volume in ml per min.
U = Urine urea conc. in mg /100 ml
P = Plasma urea conc. in mg/ 100 ml
With maximum clearance, normal is 64-99 ml/min.

If urine flow is less than 2 ml per min, the high concentration of urea in the renal tubules causes increased re-absorption. To compensate for the lower clearances observe with the reduced urine flow with a different formula is applied.

$$C_s = \frac{U\sqrt{V}}{P}$$

With standard clearance, normal value is 40-68 ml/min

Clinical Significance

The urea clearance values fall progressively with increasing renal failure. If the clearance falls below 20% it is considered to be severe renal failure. Below 5 percent uremic comas may be present.

URINE CREATININE

For the determination of urine creatinine, 24 hrs of urine sample diluted to 1:10 is used. Alkaline picrate method (serum creatinine) can be applied. If proteins are present, deproteinization of urine is must.

Calculations

$$\text{Urine creatinine mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 100$$

For the determination of 24hr creatinine excretion, measure the urine volume, and calculate the result as follows:

$$\text{Creatinine excretion, mg/24hr} = \frac{\text{urine creatinine mg/dl} \times \text{vol. of 24 hrs urine}}{100}$$

Normal Value :
Males — 19-26 mg/kg of body weight/24 hr.
Females — 14-21 mg/kg of body weight/24 hr.

Clinical Significance

The amount of creatinine excreted varies with the muscle mass and is nearly constant for individual. Increased excretion of creatinine occurs in tissue metabolism i.e. in fever. The excretion rate decreases in all kinds of renal diseases and also in postrenal conditions. Decreased excretion of creatinine also occurs in starvation, muscle atrophy and muscular weakness.

CREATININE CLEARANCE TEST

A creatinine clearance test measures how well creatinine is removed from blood by the kidneys. Compared to a blood creatinine level, a creatinine clearance test provides a more precise measure of how well the kidneys are working. A creatinine clearance test is performed both on a blood sample and on a sample of urine collected over 24 hours (24-hour urine sample).

Creatinine clearance is defined as the amount of plasma in ml, which would have to be completely cleared of the creatinine, each minute by both the kidneys in order to account for its rate of excretion.

The specimen is 24-hour urine sample. The actual period of urine collection must be accurately timed. The starting and finishing of the test of the patient with an empty bladder. The urine volume is measured and minute volume V- is calculated.

$$V = \frac{\text{Total volume of urine in ml}}{\text{Time of collection in minute}}$$

The creatinine concentration of urine (U) and plasma (P) are determined by above methods. In case of any delay in performing the test, specimen should be stored in refrigerator.

$$\text{Creatinine clearance} = \frac{U \times V}{P}$$

Normal Value:
Clearances vary with body weight. It is generally expressed as $1.73 m^2$ of the body surface area.
Males — 95 - 140 ml/min.
Females — 85 - 125 ml/min.

Clinical Significance

Clearance values are decreased in impaired renal function and so provide a rough impression of glomerular damage. It decreases in renal failure with values below 10 ml per minute in severe cases. In less severe failure cases the creatinine clearance will have fallen to about half before a raise in blood creatinine is detectable. Clearance tests are therefore of most value in the diagnosis of early renal disease They are also useful in monitoring progress, although once the serum creatinine is raised, this estimation alone will be sufficient.

URINE URIC ACID

The urine uric acid is made up of an exogenous part that is formed from purine rich diet and an endogenous part that is formed from the breakdown of nucleoproteins.

For the determination of urine uric acid, 24 hr of urine sample diluted to 1:200 is used. Henry – Caraway Method (serum uric acid) can be applied. If proteins are present, deproteinization of urine is must.

Calculations

$$\text{Urine uric acid mg/dl} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 100$$

For the determination of 24 hr uric acid excretion, measure the urine volume, and calculate the result as follows:

$$\text{uric acid excretion,mg/24 hr} = \frac{\text{urine uric acid mg/dl} \times \text{vol. of 24 hrs. urine}}{100}$$

Normal Value: $\leq$ 750 mg/24 hr.

Clinical Significance

A consistently high uric acid excretion is found in gout and leukemia. The uric acid determination is important to find out the possibility of urinary calculi (of uric acid type). The uric acid creatinine ratio is > 1.0 in most patients with acute renal failure due to hyperuricemia but lower in other causes of acute renal failure.

18 Cardiac Function Test

The cardiac function test estimates all the lipid contents of the body. The important lipid profile tests include:
Total lipids

- Serum total cholesterol
- Serum HDL cholesterol
- Serum triglycerides
- LDL and VLDL

SERUM TOTAL CHOLESTEROL

Cholesterol is a lipid and is classified as sterol. It is widely distributed in various animal tissues and vegetable oils and consumed with food. It can also be synthesized in the liver. It is a normal constituent of bile, and is principal constituent of most gallstones. It is important in metabolism serving as precursor of various steroid hormones, e.g. sex hormones and adrenal corticoids.

Serum total cholesterol includes esterified cholesterol as well as non-esterified cholesterol.

Method

I. Watson method, and
II. Enzymatic method

I. Watson Method

Specimen: Serum is preferred. Plasma can also be used. Fluoride, oxalate, or EDTA can also be used for this method.

Principle

Cholesterol reacts with acetic anhydride in the presence of glacial acetic acid and conc. sulfuric acid to form green coloured complex. Intensity of the colour is proportional to the cholesterol concentration. It is measured at 520–580 nm.

Reagent

1. Cholesterol reagent (this should be stored in amber coloured bottle at room temperature)
2. Conc. sulfuric acid
3. Cholesterol std. (200 mg/dl in glacial acetic acid)

Procedure

Cholesterol reagent is highly corrosive so, it should not be pipetted by mouth. Take three test tubes and mark them as T, S, and B. Add 2.5 ml cholesterol reagent in each tube. Add 0.1 ml of serum, 0.1 ml of cholesterol std. and 0.1 ml of distilled water in test, std. and blank respectively. This is an exothermic reaction. So, cool the tubes to room temperature by placing in water bath. Now carefully add 0.5ml of sulfuric acid to each tube mix thoroughly and keep in water bath at room temperature for 10 min. Read the absorbance of test and std. against blank at 575nm.

Reagent	*T*	*S*	*B*
Cholesterol reagent	2.5 ml	2.5 ml	2.5 ml
Serum	0.1 ml	—	—
Cholesterol std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml
Cool to room temperature			
Conc. Sulfuric acid	0.5ml	0.5ml	0.5ml

Calculations

$$\text{Serum cholesterol mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 200$$

II. Enzymatic Method

Specimen

Serum or heparinised plasma

Principle

Cholesterol esterase hydrolyses cholesterol ester into free cholesterol and fatty acids. In the second reaction, cholesterol oxidase converts free cholesterol to cholest-4en-3one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide reacts with 4- amino antipyrine and phenol to produce red colour. The intensity of red colour is directly proportional to the amount of cholesterol present in sample.

$$\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{Free cholesterol + Fatty acids}$$

$$\text{Free cholesterol} \xrightarrow{\text{cholesterol oxidase}} \text{Cholest- 4en-3one + } H_2O_2$$

$$H_2O_2 + \text{4- amino antipyrine + Phenol} \xrightarrow{\text{peroxidase}} \text{Red colour}$$

Reagent

1. Buffer/Enzymes/Chromogen
2. Phenol (30 mg/dl)
3. Cholesterol std. (200 mg/dl)

Procedure

Prepare working reagent freshly by mixing 10 ml of reagent 1. and 5ml of reagent 2. i.e, phenol. Take three test tubes and mark them as T, S, and B. Add one ml of freshly prepared working reagent in each tube. Add 0.1 ml of serum, 0.1 ml of cholesterol std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and keep at 37°C for 20 min. Read the absorbance of test and std against blank at 530 nm.

Reagent	*T*	*S*	*B*
Working reagent	1ml	1ml	1ml
Serum	0.1 ml	—	—
Cholesterol std.	—	0.1ml	—
Distilled water	—	—	0.1 ml

Calculations

$$\text{Serum cholesterol mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 200$$

Normal Value:

Neonates	45 – 100 mg %
1 – 19 yrs	120 – 240 mg %
20 – 29 yrs	144 – 275 mg %
30 – 39 yrs	165 – 295 mg %
40 – 49 yrs	177 – 350 mg %
50 – 59 yrs	160 – 330 mg %
> 69 yrs	170 – 300 mg %

Clinical Significance

Total cholesterol estimation is useful in monitoring for increased risk factor for coronary artery disease, screening and monitoring for hyperlipidemias.

It is high in hyperlipoproteinaemias, hypothyroidism, nephrosis, pancreatic disease like diabetes mellitus, chronic pancreatitis, biliary obstruction like stone, carcinoma, biliary cirrhosis, it is also increased in cholesterol ester storage disease and Von Gierke's disease. Usage of some drugs like birth control pills, amiodarone, or vitamins can also show increased cholesterol value.

The serum total cholesterol levels are decreased in severe liver cell damage, hyperthyroidism, chronic anaemia, cortisone and ACTH therapy. The levels of total cholesterol are also low in Tangier disease and in some infections.

HIGH DENSITY LIPOPROTEINS (HDL)

HDL is a lipoprotein found in the blood. It is called "good" cholesterol because it removes excess cholesterol from the blood and takes it to

the liver. A high HDL level is related to lower risk of heart and blood vessel disease.

Method

Watson Method

Specimen

Serum (fasting)

Principle

In the presence of phosphotungstic acid and magnesium chloride, LDL, VLDL and chylomicrons are precipitated. Centrifugation leaves only HDL in supernatant. Cholesterol in HDL fraction can be tested by usual method.

Reagent

1. Cholesterol reagent
2. Conc. sulfuric acid
3. PTA phosphotungstic acid reagent
4. Magnesium chloride reagent
5. Cholesterol std. (100 mg/dl)

Procedure

Part I. Precipitation of LDL and VLDL

Take 0.5 ml of serum. add 0.05 ml of PTA reagent and 0.02 ml of magnesium chloride reagent. Mix well and centrifuge at 3000 RPM for 20 min. to obtain a clear supernatant. LDL and VLDL will form precipitate. Only HDL will remain in the supernatant.

Part II. Estimation of HDL

Separate the supernatant by using Pasture pipette. Take three test tubes and mark them as T, S, and B. Add 2.5 ml cholesterol reagent in each tube. Add 0.1 ml of supernatant, 0.1 ml of cholesterol std. and 0.1 ml

of distilled water in test, std. and blank respectively. This is an exothermic reaction. So, cool the tubes to room temperature by placing in water bath. Now carefully add 0.5ml of sulfuric acid to each tube. Mix thoroughly and keep in water bath at room temperature for 10 min. Read the absorbance of test and std. against blank at 520 nm

Reagent	*T*	*S*	*B*
Cholesterol reagent	2.5 ml	2.5 ml	2.5 ml
Supernatant	0.1 ml	—	—
Cholesterol std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml
	Cool to room temperature		
Conc. sulfuric acid	0.5 ml	0.5 ml	0.5 ml

Calculations

$$\text{Serum HDL cholesterol mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 114$$

Clinical Significance

Estimation of HDL is useful in diagnosis of various lipoproteinemias and to assessment the risk for CAD(coronary heart disease). HDL is inversely proportional to CAD.

It is increased in — increased clearance of triglyceride(VLDL), insulin treatment, vigorous exercise, moderate consumption of alcohol, oral esterogen use, familial lipid disorders with the protection against arteriosclerosis, in hyperalphalipoproteinaemias and hypobetalipoproteinaemias. HDL also increases in 1 in 20 adults with mild increase in total cholesterol.

There are various secondary causes for decrease in HDL level, e.g. obesity, smoking, diabetes mellitus, stress and recent illness, lack of exercise, hypo- and hyperthyroidism, starvation (non- fasting sample is 10-15% lower). As stated earlier, HDL is inversely proportional to CAD. For every 1 mg/dl decrease in HDL, risk for CAD increases by 2- 3%.

TOTAL CHOLESTEROL/HDL RATIO

The risk associated with total cholesterol/HDL ratio is expressed as follows:

Low risk	3.3 – 4.4
Average risk	4.4 – 7.1
Moderate risk	7.1 – 11.0
High risk	> 11

VLDL AND LDL

i. VLDL: It is a major carrier of triglyceride (60 – 70 percent triglyceride, 10-15 cholesterol) circulating fatty acids are vitalized by the liver to form triglycerides that are packaged with apoprotein and cholesterol and exported into blood as very low density lipoproteins.

Normal Value = 25 – 50 %
VLDL can be estimated by Friedewald equation

$$\text{VLDL} = \frac{\text{Triglycerides}}{5}$$

This formula is applicable only when the triglycerides level is below 400 g/dl.

ii. LDL –Low Density Lipoproteins: Degradation of VLDL leads to major source of LDL. LDLs are cholesterol rich remnants of the VLDL. LDL is a lipoprotein found in the blood. It is called "bad" cholesterol because it picks up cholesterol from the blood and takes it to the cells. A high LDL level is related to a higher risk of heart and blood vessel disease. LDL is more prevalent in blood. It is finally catabolised in the liver and possibly in non-hepatic cells as well.

Normal Value: 62- 185 mg/dl

LDL is measured by ultracentrifugation and by analysis after antibody separation from HDL and VLDL. LDL can be estimated by following formula. (Friedewald equation)
LDL = Total cholesterol – (HDL cholesterol) – (VLDL)

Clinical Significance

Determination of LDL helps in assessment of risk and decide treatment for CAD.

Increase in LDL is directly related to risk of CAD. The LDL is estimated high in–Chronic renal failure, hypothyroidism, diabetes mellitus, nephritic syndrome, Wolman's disease, etc.

LDL is low in severe illness, abetalipoproteinemia and oral estrogen use.

TOTAL LIPIDS

Lipids are usually defined as those components that are soluble in organic solvents (such as ether, hexane or chloroform), but are insoluble in water. This group of substances includes triacylglycerides, cholesterol, LDL and VLDL, HDL lipoproteins, free fatty acids, phospholipids, sterols, and carotenoids.

Method

Sulfo-phoshovanillin

Specimen

Serum (fasting)

Principle

Lipids react with vanillin in the presence of sulfuric acid and phosphoric acid to form a pink coloured complex. This is measured at 546 nm.

Reagent

1. Total lipid std. (1000mg/dl)
2. Phoshovanillin (colour reagent)
3. Conc. sulfuric acid

Reagent: 1. and 2. should be kept in refrigerator in amber colour bottle.

Procedure

Take two test tubes. Add the reagents as follows:

Reagent	*T*	*S*
Total lipid std.	—	0.05ml
Serum	0.05ml	—
Conc. sulfuric acid	2.0 ml	2.0 ml

Mix thoroughly. Cotton plug it. Keep in a boiling water bath for 10 min. Then cool the tubes and pipette into dry test tubes as follows.

Reagent	*T*	*S*	*B*
From above solution	0.10 ml	0.10 ml	—
Conc. sulfuric acid	—	—	0.10 ml
Colour reagent	2.5 ml	2.5 ml	2.5 ml

Mix thoroughly and keep at room temperature for 15 min. Read the absorbance of test and std. against blank at 546 nm.

Calculations

$$\text{Serum Total lipids mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 1000$$

Normal value : 400 – 1000 mg/dl

SERUM TRIGLYCERIDES

Triglycerides are a type of fat found in the blood. The blood level of this type of fat is most affected by the foods one eat (such as sugar, fat or alcohol) but can also be high due to being overweight, having thyroid or liver disease and genetic conditions. High levels of triglycerides are related to a higher risk of heart and blood vessel disease.

Method

GPO POD / Enzymatic method

Specimen

Serum /plasma (heparin or EDTA). Preferably fasting

Principle

Triglycerides are hydrolysed by lipase to glycerol and fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to Glycerol-3-phosphate(G-3-P) which is oxidized by enzyme Glycerol-3-phosphate oxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-amino antipyrine and P- chlorophenol in presence of enzyme peroxidase (POD) to produce a red quinoneimine dye.The intensity of red colour is directly proportional to the amount of cholesterol present in sample.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G-3-P} + \text{ADP}$$

$$\text{G-3-P} + O_2 \longrightarrow H_2O_2 + \text{Dihydroxyacetone phosphate}$$

$$H_2O_2 + \text{4- amino antipyrine} + \text{P- chloro phenol} \xrightarrow{\text{perioxidase}} \text{Red colour} + H_2O$$

Reagent

1. Buffer/Enzymes/Chromogen
2. P-chlorophenol
3. Triglyceride std (100 mg/dl)

Procedure

Prepare working reagent freshly by mixing 10 ml of reagent 1. and 5ml of reagent 2. i.e, P- chlorophenol. Take three test tubes and mark them as T, S, and B. Add one ml of freshly prepared working reagent in each tube. Add 0.1 ml of serum, 0.1 ml of triglyceride std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and keep at 37°C for 20 min. Read the absorbance of test and std. against blank at 530 nm.

Reagent	*T*	*S*	*B*
Working reagent	1 ml	1 ml	1 ml
Serum	0.1 ml	—	—
Triglyceride std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml

Calculations

$$\text{Serum Triglyceride mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 100$$

Normal value : 80 – 150 mg/dl

19 Electrolytes

SODIUM

Sodium is a major extracellular cation (Na^+) of the body. Sodium salts are necessary to preserve a balance between $Ca^{++}$and K^+ to maintain normal heart action and equilibrium of the body. Sodium salts regulate the osmotic pressure in the cells and fluids and guard against an excessive loss of water from the tissues. Almost all blood sodium is found in the plasma. There is very little in the red cells.

Method

Modified Maruna and Trinder's method.

Specimen

Serum

Principle

Sodium from the specimen is quantitatively precipitated as the triple salt uryl magnesium sodium acetate and the excess of uryl salt reacts with potassium ferrocyanide to produce brown colour. The intensity of brown colour produced is inversely proportional to the sodium conc. of the specimen.

Reagent

1. Std. sodium chloride solution (equivalent to 300 mg of Na)
2. Uranlymagnesium acetate solution
3. Acetic acid 1 % aq. solution
4. Potassium ferrocyanide 20% solution

Procedure

Part I. Precipitation Step

Take three test tubes and mark them as T, S, and B. Add five ml of Uranylmagnesium acetate solution in each tube. Add 0.1 ml of serum, 0.1 ml of sodium std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and allow to stand for 5 min. Centrifuge for one min. at 3000 rpm. to get clear supernatant.

Reagent	*T*	*S*	*B*
Uranlymagnesium acetate solution	5 ml	5 ml	5 ml
Serum	0.1 ml	—	—
Sodium std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml

Part II. Colour Formation

Take three test tubes and mark them as T, S, and B. Add 0.2 ml of supernatant from Part I. in respective tubes. Add 8 ml of acetic acid in each tube. Now add 0.2 ml of potassium ferrocyanide in each tube. Now make up the volume to 10 ml by acetic acid. Read the absorbance of test and std. against blank at 480 nm. Be sure that the readings are taken within 10 min of last step.

Reagent	*T*	*S*	*B*
Supernatant from Part I (T)	0.2 ml	—	—
Supernatant from Part I (S)	—	0.2 ml	—
Supernatant from Part I (B)	—	—	0.2 ml
Acetic acid	8 ml	8 ml	8 ml
Potassium ferrocyanide	0.2 ml	0.2 ml	0.2 ml
Acetic acid	1.6 ml	1.6 ml	1.6 ml

Calculations

$$\text{Serum sodium mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 300$$

SERUM POTASSIUM

Unlike sodium, potassium is the major intracellular cation of the body. Within the cells it plays important role in maintenance of acid-base

balance, osmotic pressure and water retention. Intracellular potassium is essential for several important metabolic reactions catalyzed by enzymes. It is also very important constituent of the extracellular fluid because it influences muscle activity notably the cardiac muscle.

Method

Turbidometric method:

Specimen

Serum

Principle

Potassium ions from specimen react with sodium tetraphenyl boron resulting in a turbid suspension. The extent of turbidity is measured photometrically at 620 nm is proportional to the potassium concentration.

Reagent

1. Potassium reagent
2. Potassium Std. (5 mol / lit.)

Procedure

Take two test tubes and mark them as T, and S. Add three ml of potassium reagent in each tube. Add 0.1 ml of serum, and 0.1 ml of potassium std. in test, and std. respectively. Mix well and allow to stand for 5 min at room temperature. Read the absorbance of T and S against reagent at 620 nm.

Reagent	*T*	*S*
Potassium reagent	3 ml	3 ml
Serum	0.1 ml	—
Sodium std.	—	0.1 ml

Another popular method for determination of sodium and potassium is flame photometry—

Reagent

1. Stock standard for sodium (1000 mEq/l)
2. Stock standard for potassium (100 mEq/l)

Mixed working standards are prepared as follows:

1. Sodium/potassium (120/2.0 mEq/l): It contains 120 mEq/l of sodium and 2.0 mEq of potassium in one liter distilled water. It is prepared by mixing 12 ml Stock standard for sodium and 2 ml Stock standard for potassium in 86 ml of distilled water.
2. Sodium/potassium (140/4.0 mEq/l): It contains 140 mEq/l of sodium and 4.0 mEq of potassium in one liter distilled water. It is prepared by mixing 14 ml stock standard for sodium and 4 ml stock standard for potassium in 82 ml of distilled water.
3. Sodium/potassium (160/6.0 mEq/l): It contains 160 mEq/l of sodium and 6.0 mEq of potassium in one liter distilled water. It is prepared by mixing 16 ml stock standard for sodium and 6 ml Stock standard for potassium in 78 ml of distilled water.

Note: mEq/day = milliequivalents per day

Specimen

Serum or heparinised plasma

FLAME PHOTOMETER

It is a spectrophotometer in which a spray of metallic salts in solution is vaporized in a very hot flame and subjected to quantitative analysis by measuring the intensities of the spectral lines of the metals present.

1. Main unit and
2. Compressor unit are the important components of the equipment.

1. Main Unit

It consists of —a. An atomizer b. Mixing chamber c. Burner d. Optical filters e. Photo detectors f. Two digital displays g. Air regulator h. Gas regulator i. Gas pressure gauge.

2. Compressor Unit

It delivers oil free compressed air to the atomizer.

The atomizer and flame are the most important components in the flame photometer. The function of atomizer is to break up the solution into fine droplets so that the atoms will absorb heat energy from the flame and becomes excited.

The gases used for the flame photometer are 1. a mixture of hydrogen and oxygen 2. natural gas 3. acetylene and propane with air or oxygen 4. LPG (liquid petroleum gas).

Principle

The solution under test is passed carefully, under controlled conditions as a very fine spray in the air supply to non-luminous flame. In the flame the solution evaporates and the salt dissociates to give natural ions, which emit light of the characteristic wavelength. The flame is simultaneously monitored by both the channel consists of a detector which views the flame through a narrow band optical filter. The photo detector out puts are connected to two independent digital displays, which are calibrated for direct concentration readouts. Initial calibration is done by using at least three standards of different concentrations.

Procedure

Take four test tubes. Mark them as Test, std.1, std.2, and std.3. to each tube add 10 ml of distilled water. Add 0.1 ml of serum to test. Add 0.1 ml of std. (120/2.0 mEq/ l), 0.1 ml of std. (140/4.0 mEq/ l), 0.1 ml of std. (160/6.0 mEq/ l), to std.1, std.2, and std.3 respectively.

Reagent	*Test*	*Std.1*	*Std.2*	*Std.3*
Distilled water	10 ml	10 ml	10 ml	10 ml
Serum	0.1 ml	—	—	—
Std. (120 / 2.0 mEq/ l)	—	0.1 ml	—	—
Std. (140 / 4.0 mEq/ l)	—	—	0.1 ml	—
Std. (160 / 6.0 mEq/ l)	—	—	—	0.1 ml

Put on the main switch. Put on air compressor and adjust the required air pressure, by adjusting the knob meant for air. Introduce the distilled water through atomizer. Put on gas and control the flame by adjusting the knob meant for gas till the flame is divided into five

sharp cones. Adjust the proper filters for the simultaneous determination of sodium and potassium. Make zero adjustment by using distilled water. Introduce the std. 120 /2.0 mEq/l and by using the knob meant for sodium the digits 120.0 and by using the knob meant for potassium the digits 2.0. are adjusted. Introduce the std. 140/4.0 mEq/l. If the standards are accurately prepared the digital display will indicate exact concentration for both sodium and potassium. Introduce the std. 160/6.0 mEq/l and confirm the accuracy of the standard. Now introduce the test and record the readings for sodium and potassium.

Normal value:

Solium (Na) — 135 - 145 mEq/ l

Potassium (K) — 1 - 15 yr —— 3.7 - 5.0 mEq/ l
16 - 59 yr —— 3.6 - 4.8 mEq/ l
≥ 60 yr —— 3.9 - 5.3 mEq/ l

Clinical Significance

Estimation of serum sodium is useful in diagnosis and treatment of dehydration and over hydration. Changes in sodium more often reflect changes in water balance. Increased sodium values (hypernatremia) are observed in conditions such as —

1. Severe dehydration
2. Diabetes insipidus
3. Salt poisoning
4. Cushing's syndrome
5. In certain post-renal conditions like enlarged prostate leading to obstruction of urine flow.

Decreased sodium values (hyponatremia) are observed in conditions such as —

1. Severe prolonged diarrhoea and vomiting
2. Salt losing nephritis, and
3. Addison's disease.

Estimation of serum potassium is very useful in paralysis, severe fluid and electrolyte loss, diabetic coma, renal failure, etc. Increased potassium values (hyperkalemia) are observed in conditions such as

1. Addison's disease
2. Renal glomerular disease

3. In anuria and oliguria
4. Familial hyperkalemic paralysis
5. Acute acidosis
6. Decreased insulin, and
7. Intravascular haemolysis.

Decreased potassium values (hypokalemia) are observed in conditions such as —

1. Cushing's syndrome
2. Renal tubular damage
3. Metabolic alkalosis, and
4. Malnutrition.

URINE SODIUM AND POTASSIUM

I. *Urine sodium:* To determine urine sodium value, the method used is flamephotometry. The procedure is exactly same as that of serum sodium. Use undiluted urine instead of serum.

Calculation

24 hr excretion of urine sodium =

$$\frac{\text{24 hr urine volume (ml)}}{100}$$

Normal Value: Urine sodium: 40-220 mEq/24 hr

Increased in diuretics, high sodium diet, acute tubular necrosis (ATN), salt-losing nephritis, Addison's disease, hypothyroidism, SIADH *(Syndrome Inappropriate ADH Secretion),*CHF and liver failure.

Decreased in fasting**,** some fevers, and chronic nephritis.

II. *Urine potassium :* To determine urine potassium value, the method used is flamephotometry. The procedure is exactly same as that of serum potassium. Use diluted urine (1: 10) instead of serum.

Calculation

Urine potassium, mEq/l = reading × 10

24 hrs. urine excretion =

$$\frac{\text{Urine potassium mEq/l} \times \text{24 hrs urine volume (ml)}}{1000}$$

Normal

A. Urine Potassium: 25-100 mEq/24 hr

Increased in primary or secondary aldosteronism, glucocorticoids, alkalosis, renal tubular acidosis, excess potassium intake

Decreased in acute renal failure, potassium sparing diuretics, diarrhea, and hypokalemia.

SERUM CHLORIDE

Chloride is the major extracellular anion of the body. Its primary role in the body is to maintain proper water distribution, osmotic pressure and normal anion-cation balance in the plasma. In gastric juice, chloride also plays important role in the production of HCl. The chloride ions are ingested through the food (regular salt) and filtered or reabsorbed by the kidney as per the body need.

Method

Modified Schoenfeld and Lewellen's method

Specimen

Serum or heparinised plasma

Principle

Chloride ions reacts with mercuric thiocynate to form mercuric chloride, an undissociated salt to liberate thiocynate ions. These thiocynate ions reacts with the ferric ions to form ferric thiocynate, which is coloured compound. The colour formed is proportional to the chloride content of the specimen. The absorbance can be read at 520 nm. The final colour is stable for half an hour:

$$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$$

$$3\,SCN + Fe^{3+} \longrightarrow Fe(SCN)_3 \text{ (coloured compound.)}$$

Reagent

1. Chloride reagent
2. Chloride std (100 mEq/l)

Procedure

Take three test tubes and mark them as T, S, and B. Add two ml of chloride reagent in each tube. Add 0.1 ml of serum, 0.1 ml of chloride std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and keep at room temperature for 2 min. Read the absorbance of test and std. against blank at 505 nm.

Reagent	*T*	*S*	*B*
Chloride reagent	1 ml	1 ml	1 ml
Serum	0.1 ml	—	—
Chloride std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml

Calculations

$$\text{Serum chloride mEq/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 100$$

Normal Value: 96 – 109 mEq/dl

Clinical Significance

Serum chloride is very useful to assess electrolyte, acid-base and water balance. Serum chloride is increased in metabolic acidosis associated with prolonged diarrhoea, renal tubular diseases, respiratory alkalosis, some cases of hyperparathyroidism, diabetes insipidus, dehydration, and in conditions causing decreased renal blood flow, i.e. congestive heart failure.

Serum chloride levels are decreased in prolonged vomiting (loss of HCl), salt losing renal diseases, chronic respiratory acidosis, burns, and effect of certain drugs like-corticosteroids, bicarbonates, etc.

URINE CHLORIDE

Urine chloride: To determine urine chloride value, the method used is modified Schoenfeld and Lewellen's method. The procedure is exactly same as that of serum chloride. Use undiluted urine instead of serum.

Calculation

$$\text{24 hr urine excretion} = \frac{\text{Urine chloride mEq/l} \times \text{24 hr urine volume (ml)}}{1000}$$

Normal Values

The normal range is 20 to 250 mEq/day. This range is highly dependent on salt intake and the state of the individual's hydration.

Clinical Significance

Increased urine chloride excretion may be caused by—Increased salt intake, postmenstrual diuresis, pharmacologic diuresis, salt-losing nephritis, adrenocortical insufficiency.

Decreased urine chloride excretion may occur with—Decreased salt intake, adrenocortical hyperfunction, Extrarenal fluid loss (such as diarrhoea, vomiting, sweating, and gastric suction), salt retention.

SERUM PHOSPHORUS

Most of the phosphorus in the blood exists as inorganic phosphate. About 80 percent of the total phosphorus is combined with calcium in bones and teeth. It is found in every cell of the body. About 10 percent is combined with proteins, lipids and carbohydrate and other compounds in blood and muscle. The remaining 10 percent is widely distributed in various chemical compounds.

Method

Gomorri's method.

Principle

Protein in serum is first removed by treating with TCA . Protein-free filtrate is then treated with an acid molybdate, which reacts with inorganic phosphate to form phosphomolybdic acid. The colour reagent, metol reduces phosphomolybdic acid to give a blue coloured compound. The intensity of the colour is measured at 660 nm.

Reagents

1. Trichloroacetic acid (10 g/dl)
2. Molybdate reagent
3. Colour reagent, Metol
4. Phosphorus std. (5 mg/dl)

Procedure

Take two centrifuge tubes. Mark them as test and diluted std. add 4.5 ml TCA reagent in each tube. Add 0.5 ml of serum in test and 0.5 ml of std. in diluted std. tubes. Mix and centrifuge to get clear filtrate. Pipette in the tubes as follows.

	Test	*Std.*	*Blank*
Filtrate	2.5 ml	—	—
Diluted std	—	2.5 ml	—
Distilled water	—	—	2.5 ml
Molybdate reagent	0.5 ml	0.5 ml	0.5 ml
Colour reagent	0.5 ml	0.5 ml	0.5 ml

Mix thoroughly and keep in the dark for 10 min. Read the intensities at 660 nm.

Calculation

$$\text{Serum inorganic phosphorus (mg/dl)} = \frac{\text{OD of T}}{\text{OD of S}} \times 5$$

Normal Value:

Neonates	45 – 100 mg %
1 – 19 yr	120 – 240 mg %
20 – 29 yr	144 – 275 mg %

30 – 39 yr	165 – 295 mg %
40 – 49 yr	177 – 350 mg %
50 – 59 yr	160 – 330 mg %
> 69 yr	170 – 300 mg %

Clinical Significance

Decreased serum phosphorus values are observed in preliminary hyperparathyroidism, rickets (vitamin D deficiency) and in Fanconi's syndrome (defect in re-absorption of phosphorus). Increased serum phosphorus levels are found in hypervitaminosis-D, hypoparathyroidism and in renal failure.

URINE INORGANIC PHOSPHORUS

The daily excretion of inorganic phosphorus on an average diet is about one gram. There is increased excretion of phosphorus in urine in hyperparathyroidism, and it is reduced in hypoparathyroidism. Phosphate excretion is also reduced in rickets, due to impaired absorption of phosphorus.

Method

Gomorri's method.
Reagents and principle are same as that of serum inorganic phosphate.

Specimen

24 hr urine sample with thymol crystals added as a preservative.

Procedure

Dilute the urine sample to 1: 100 in TCA reagent. Also dilute the std. 5 mg/dl in TCA reagent. If proteins are present, a preparation of protein free filtrate is must, if proteins are absent then directly proceed for the part II step of the serum inorganic phosphate procedure.

Calculation

$$\text{Urine inorganic phosphorus (mg/dl)} = \frac{\text{OD of T}}{\text{OD of S}} \times 5$$

$$\text{24 hr urine excretion} = \frac{\text{Urine phosphorus mg/dl} \times \text{24 hr urine volume (ml)}}{100}$$

Clinical significance

High urinary phosphorus (i.e. increased renal losses) occurs in primary hyperparathyroidism, vitamin D deficiency, renal tubular acidosis, diuretic use. Phosphates are among the substances, which may be lost in the Fanconi syndrome. Renal loss of phosphate may itself lead to rickets or osteomalacia.

Low in hypoparathyroidism, pseudohypoparathyroidism, vitamin D intoxication.

SERUM CALCIUM

Calcium is the major constituent of bone. Calcium in serum is present in ionized form or as a complex with protein or other inorganic substances like citrate, phosphate and others. Calcium plays many important roles in physiology of the body like it activates many enzymes and plays a key role in blood coagulation.

Method

OCPC method

Specimen

Serum or heparinised plasma. It should be separated as soon as possible.

Principle

Calcium in an alkaline medium reacts with O-cresolphthalein complexone (OCPC) to form an intense chromophore, which is of purple colour. Read the absorbance at 575 nm.

Reagent

1. O-cresolphthalein complexone reagent
2. Buffer solution
3. Calcium std. (10 mg/dl)

Procedure

First of all prepare working solution by mixing equal amounts of reagent 1. and reagent 2. This is to be freshly prepared as it is stable only for one day. Take three test tubes and mark them as T, S, and B. Add six ml of freshly prepared working reagent in each tube. Add 0.05 ml of serum, 0.05 ml of calcium std. and 0.05 ml of distilled water in test, std. and blank respectively. Mix well and keep at room temperature for exactly 10 min. Read the absorbance of test and std. against blank at 575 nm.

Reagent	*T*	*S*	*B*
Working reagent	6 ml	6 ml	6 ml
Serum	0.05 ml	—	—
Calcium std.	—	0.05 ml	—
Distilled water	—	—	0.05 ml

Calculations

$$\text{Serum calcium mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 10$$

Normal value:

1-3 yr	8.7-9.8 mg/dl
4-11 yr	8.8-10.1 mg/dl
12-13 yr	8.8-10.6 mg/dl
14-15 yr	9.2-10.7 mg/dl
> 16 yr	8.9-10.7 mg/dl

Clinical Significance

Determination of serum calcium level is useful in diagnosis of parathyroid dysfunction, hypercalcemia of malignancy, 90 percent of cases of hypercalcemia are due to hyperparathyroidism, neoplasms or

granulomatous diseases. Hypercalcemia of sarcoidosis adrenal insufficiency and hyperthyroidism tend to be found in clinically evident disease. Blood calcium should be monitored in renal disease, effects of various drugs, acute pancreatitis, postoperative thyroidectomy, and parathyroidectomy.

Calcium levels are found to be low in hypoparathyroidism, malabsorption of calcium and vitamin D, chronic renal disease with uremia, bone disease, late pregnancy, asphyxia, infants of diabetic mothers, cerebral injuries, malignant disease, etc.

URINE CALCIUM

The same method can be used to determine urine calcium.

Specimen

24 hr of urine sample preserved with thymol crystals.

Calculations

$$\text{Urine Calcium mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 10$$

For the determination of 24 hr calcium excretions, measures the urine volume, and calculate the result as follows:

$$\text{Calcium excretion, mg/24 hrs} = \frac{\text{Urine calcium mg/dl} \times \text{vol. of 24 hr urine}}{100}$$

Normal Value: 100 – 300 mg/dl in 24 hr of urine sample.

Clinical Significance

Determination of urine calcium level is useful in diagnosis of hypercalciuria causing renal calculi. High calcium levels in urine are seen in – hyperparathyroidism, excess milk intake, high calcium diet, rapidly progressive osteoporosis, multiple myeloma, Paget's disease, etc. hypercalciuria without hypercalcemia are due to medullary sponge kidney, renal tubular acidosis, hyperthyroidism , etc.

Low calcium level in urine is due to renal failure, hypoparathyroidism, rickets, osteomalacia, metastatic carcinoma of prostate, etc.

20 Enzyme Assay

AMYLASE

Amylase is a hydrolytic enzyme that splits complex carbohydrates such as starch and glycogen into simpler molecules of sugars. (e.g. glucose, maltose). Serum amylase is composed of pancreatic and salivary type of isoamylases.

$$\text{Starch} + \text{Water} \xrightarrow{\text{Amylase}} \text{Sugar(s)}$$

The amylases normally occurring in human plasma are small molecules. Amylase is the only plasma enzyme normally found in the urine.

Method

I. Iodometric method
II. Visible kinetic method

I. Iodometric Method

Specimen

Unhaemolysed serum/diluted urine (1: 100)

Principle

The enzyme amylase degrades starch into dextrins and maltose. This reaction should be carried out under defined conditions. When end products are treated with iodine (colour reagent) decrease in blue colour is observed, comparing to that produced with blank. The disappearance of blue colour is directly proportional to the amylase concentration in the specimen.

Reagent

1. Buffered substrate
2. Stock colour reagent

Procedure

Dilute stock colour reagent to 1:10 with distilled water to get working colour reagent. Take two test tubes. Label it as test and control. Add 2.5 ml of buffered substrate in each tube . Keep at 37°C for 5 min. Add 0.1ml serum in test. Mix and incubate it for 7 min. Add 2.5 ml of working colour reagent in each tube. Now add 0.1 ml serum in control. At last add 20 ml of distilled water in both the tubes. Mix thoroughly and read the absorbance of test and std. against distilled water at 660 nm.

Reagent	*T*	*C*
Buffered substrate	2.5 ml	2.5 ml
Keep at 37°C for 5 min		
Serum	0.1 ml	—
Mix and incubate it for 7 min		
Working colour reagent	2.5 ml	2.5 ml
Serum	—	0.1 ml
Distilled water	20 ml	20 ml

Calculation

The serum amylase is expressed in Caraway units. One Caraway unit is the amount of enzyme that will hydrolyse 10 mg of starch in 30 min to a colourless stage.

$$\text{Serum amylase in Caraway unit} = \frac{\text{OD of C} - \text{OD of T}}{\text{OD of C}} \times 400$$

II. Visible Kinetic Method

Principle

Alpha-amylase hydrolyzes p-nitrophenyl-alpha-D-maltoheptoside ($PNPG_7$) to p-nitrophenylmaltotriose ($PNPG_3$) and maltotertrose. Glucoamylase hydrolyses $PNPG_3$ to p-nitrophenylglycoside ($PNPG_1$) and glucose $PNPG_1$ is hydrolyzed by α- glucosidase to glucose and

p-nitrophenol, which produces yellow colour. The rate of increase in yellow colour is proportional to α- amylase activity in the sample and is measured at 405 nm.

$$PNPG_7 \xrightarrow{\alpha\text{ - amylase}} PNPG_3 + \text{maltotertrose.}$$

$$PNPG_3 \xrightarrow{\text{Glucoamylase}} PNPG_{1\,+}\ \text{glucose}$$

$$PNPG_1 \xrightarrow{\alpha\text{- glucosidase}} \text{p-nitrophenol + glucose}$$

Reagent

Amylase reagent — the lyophilized reagent is reconstituted with volume of distill water stated on the vial label. This working reagent is stable for one month in refrigerator.

Specimen

Unhaemolysed serum/diluted urine (1: 100)

Procedure

Take one ml of working amylase reagent in a test tube. Add 0.02 ml of serum and mix well. Read absorbance every 30 seconds for 2 min. Determine the absorbance difference (ΔA)/min.

Calculations

Serum amylase, IU = $\Delta A \times 7123$

Normal value:
In urine — 50 – 300 IU / hr. for 24 hr.
In serum — Below 18 yr 0 – 260 U/L
18 and above 18 yr 35 – 115 U/L

The serum amylase levels are high in acute pancreatitis, obstruction of pancreatic duct by – stone/carcinoma, biliary tract disease, acute cholecystitis complications of pancreatitis, pancreatic trauma, altered GI tract permeability, acute alcohol ingestion. Serum amylase level is also increased in salivary gland disease (like mumps), malignant tumors

specially of pancreas, lung, ovary, oesophagus, breast and colon, advanced renal insufficiency. The other causes include – chronic liver disease, ovarian cyst, diabetic ketoacidosis, splenic rupture. Increased serum amylase with low urine amylase may be seen in renal insufficiency and macroamylasemia.

Decreased serum amylase levels are clinically significant in marked destruction of pancreas, severe liver damage like in hepatitis, severe burns etc.

SERUM LIPASE

Lipases are the enzymes that hydrolyze glycerol esters of long chain fatty acids. It is fully active in presence of bile salts and a cofactor, called colipase. Most of the lipase in the serum is produced in the pancrease, but some is also secreted by the lingual glands, and gastric, pulmonary, and intestinal mucosa. Lipase activity is also seen in leucocytes, adipose tissue cells and milk. Lipase is filtered at the glomeruli but completely reabsorbed by the tubules. Therefore, it is not normally detected in urine.

Method

Colorimetric

Principle

Serum pancreatic lipase acts on a natural type of substrate, 1,2-diglyceride to liberate 2-monoglyceride. The 2-monoglyceride is hydrolyzed by monoglyceride lipase (MGLP) to produce glycerol and fatty acid. Glycerol kinase (GK) then acts on the glycerol to produce glycerol-3-phosphatase which is converted to dihydroxyacetone phosphate and hydrogen peroxide in a reaction catalyzed by glycerol-3-phosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine sodium salt (TOOS) in a reaction catalyzed by peroxidase (POD) to yield a quinone dye. The rate of increase in absorbance at 550 nm is directly proportional to the lipase activity of the sample.

1, 2-diglyceride + H_2O —— Pancreatic Lipase ⟶ 2-monoglyceride + fatty acid

2-monoglyceride + H_2O —— MGLP ————> glycerol + fatty acid

glycerol + ATP —— GK————> glycerol-3-phosphatase + ADP

glycerol-3-phosphatase + O_2 —— GPO ————> dihydroxyacetone phosphate + H_2O_2

$2H_2O_2$ + 4-aminoantipyrine + TOOS —— POD ————> quinone dye + 4 H_2O_2

Reagent

1. Lipase substrate
2. Lipase substrate buffer
3. Lipase activator

Reagent Preparation

Reconstitute the lipase substrate vial with the amount of lipase substrate buffer indicated on the vial label. Swirl to dissolve. Reconstitute the lipase standard with 3 ml of distilled water.

Specimen

Fasting, non-hemolyzed serum is the preferred.

Procedure

Take four test tubes. Label them as “Blank”, “Standard”, “Control”, “Test”, etc. Pipette 3 ml of reconstituted lipase substrate reagent to all tubes.Pipette 0.05 ml of distilled water to the blank tube and 0.05 ml of the appropriate sample to the tubes labeled “Standard”, “Control”, etc. Mix each tube well and incubate for 3-5 minutes at 37°C.

After the pre-incubation, add 1ml of Lipase activator to the blank tube. Mix well and incubate for 3 minutes at 37°C. Then measure the rate of increase in absorbance per minute at 550 nm (540-560 nm). Repeat this step for all tubes.

Reagent	*T*	*S*	*B*	*C*
Lipase substrate	3 ml	3 ml	3 ml	3 ml
Distilled water	—	—	0.05 ml	—
Serum	0.05 ml	—	—	—
Std.	—	0.05 ml	—	—
Control serum	—	—	—	0.05 ml
Mix each tube well and incubate for 3-5 minutes at 37°C.				
Lipase activator	1 ml	1 ml	1 ml	1 ml

Calculation

$$\text{Serum Lipase} = \frac{\Delta A \text{ Sample} - \Delta A \text{ Blank}}{\Delta A \text{ Standard} - \Delta A \text{ Blank}} \times \text{Conc of std.}$$

Normal values: 56 – 200 IU

Clinical Significance

Serum lipase is recognized as an important indicator for the diagnosis, and therapeutic monitoring, of pancreatic diseases. The lipase test is most often used in evaluating inflammation of the pancreas (pancreatitis), but it is also useful in diagnosing kidney failure, intestinal obstruction, mumps, and peptic ulcers. Doctors often order amylase and lipase tests at the same time to help distinguish pancreatitis from ulcers and other disorders in the abdomen. If the patient has acute (sudden onset) pancreatitis, the lipase level usually rises somewhat later than the amylase level—about 24–48 hours after onset of symptoms—and remains abnormally high for 5–7 days. It is also increased in obstruction of pancreatic duct by stone, intestinal infarction, after organ transplant, chronic liver diseases. 2–3 times increase in serum lipase is found in 80 percent patients with acute and chronic renal failure. Alcoholism, diabetic ketoacidosis, increases in lipase.

No clinically significant role has been known in which serum lipase activity is lower.

SERUM LACTATE DEHYDROGENASE (LDH)

Lactate dehydrogenase activity is present in all cells of the body. It is found in the cytoplasm of the cell. It is present in high concentration

in heart, kidney, erythrocytes and skeletal muscle. Many of the tissues show different isoenzymes composition. LDH catalyses following reaction.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + H^+$$

The optimum reaction conditions are at 37°C temperature and 8.8 – 9.8 pH.

Method

King's method

Specimen

Strictly unhaemolysed serum since, RBCs are rich in LDH .

Principle

LDH catalyses following reaction.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + H^+$$

The products formed react with 2, 4-dinitrophenyl hydrazine (DNPH) to give corresponding hydrazone. Hydrazone gives brown colour in alkaline medium, which is measure of LDH.

Reagent

1. Glycine reagent
2. Buffered substrate
3. NAD solution
4. NADH solution
5. DNPH reagent
6. 0.4 N NaOH

Procedure

Part I. Preparation of Standard Curve

Take seven test tubes. Mark them as Blank, 1, 2, 3, 4, 5, and 6 for

enzyme activity—0, 167, 333, 500, 667, 883, and 1000 respectively. Add the reagent as follows:

Reagent	*Blank*	*1*	*2*	*3*	*4*	*5*	*6*
S. LDH activity, IU	0	167	333	500	667	883	1000
NADH solution	0 ml	0.05 ml	0.10 ml	0.15 ml	0.20 ml	0.25 ml	0.30 ml
Pyruvate	0 ml	0.05 ml	0.10 ml	0.15 ml	0.20 ml	0.25 ml	0.30 ml
Buffered substrate	1.0 ml	0.9 ml	0.8 ml	0.7 ml	0.6 ml	0.5 ml	0.4 ml
NAD solution	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Distilled water	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DNPH reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Mix each tube well and keep at room temperature for 15 minutes.							
0.4 N NaOH	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml

Mix well and keep at room temperature for exactly 10 min. measure the intensities of all the tubes setting against blank at 445nm. Draw a graph by plotting OD on Y-axis and IU on X-axis.

Part II. Preparation of Test

For sample, take two test tubes - label it as test and control. Add the reagent as follows.

Reagent	*T*	*C*
Buffered substrate	1.0 ml	1.0 ml
incubate for 5 minutes at 37°C		
NAD solution	0.2 ml	0.2 ml
Serum	0.02 ml	—
Mix well and incubate for 15 minutes at 37°C.		
DNPH reagent	1.0 ml	1.0 ml
Serum	—	0.02 ml
Mix well and keep at room temperature for 15 minutes		
0.4 N NaOH	10 ml	10 ml

Calculation

Net OD of test = OD of T – OD of C

From the standard graph calculate the serum LDH value.

Normal value:

Newborn	—	160 – 1500 U/L
Infant	—	150 – 360 U/L
Child	—	150 – 300 U/L
Adult	—	100 – 250 U/L

Clinical Significance

SLDH is very non-specific test. It can be used as marker of haemolysis in anemia.

It is increased in congestive heart failure, acute myocarditis, rheumatic fever, in liver diseases like cirrhosis, obstructive jaundice, hepatitis. In lung disease like pulmonary embolus and infarction. SLDH is increased in 50 percent of patients with carcinomas specially in advanced stages. In muscle disease, in renal diseases like in nephritic syndrome, in acute pancreatitis, hypothyroidism. LDH is increased in various infections and parasitic diseases.

The only condition in which LDH is known to be decrease is X-ray irradiation.

PHOSPHATES

Phosphates belong to the class of enzymes called hydrolyses.

Phosphates are able to hydrolyze a large variety of organic phosphate esters with the formation of an alcohol and phosphate ion.

Phosphates of diagnostic importance are of two kinds- alkaline phosphates and acid phosphates. These are differentiated by their reaction in alkaline and acidic medium. The pH for measuring the alkaline phosphates activity is 10 and for acid phosphates it is 5.

Specimen

Serum is preferred, but heparinized plasma can also be used other anticoagulants inhibit the enzyme activity. Overnight fasting serum is preferred; store the serum in refrigerator if immediate analysis is not possible.

i. Alkaline Phosphates : As is described earlier .

ii. Acid Phosphatase:

A total acid phosphatase includes all phosphatase with optimum activity in the range 4 to 6. ACP is present in lysosomes of all the cells, except erythrocytes. Extralysosomal ACPs are also present in many cells. ACP activity is mainly observed in bone marrow, liver spleen, milk, platelets and highest in prostate glands.

The ACPs are unstable above pH 7.0 and temperature 37°C. More than 50 percent of ACP activity is lost in 1hour at room temperature. Acidification of the serum specimen to a pH below 6.5 aids in stabilizing the enzyme.

Method

Gutman and Gutman.

Principle

Acid phosphatase from serum converts phenyl phosphatase to inorganic PO_4 and phenol at pH 4.9. Phenol in alkaline medium reacts with 4-aminoantipyrine in presence of potassium ferricyanide forms orange red coloured complex. The colour intensity is directly proportional to enzyme activity.

$$\text{Phenyl phosphatase} + \text{Acid phosphatase} \longrightarrow \text{Phenol} + \text{inorganic } PO_4$$

$$\text{Phenol} + \text{4-aminoantipyrine} \xrightarrow[OH^-]{\text{K ferricyanide}} \text{Orange Red Complex}$$

Since tartarate inhibits prostatic fraction of enzyme, the difference in acid phosphatase activity with and without tartarate represents activity and prostratic fraction.

Reagents

1. Buffered substrate at pH4.9
2. 0.5N NaOH

3. 0.5N $NaHCO_3$
4. 4-aminoantipyrine
5. Potassium ferricyanide
6. 1M tartarate
7. Phenol std.

Procedure

Take 5 test tubes, mark them as blank, std, control, test and tartarate stable.
Add the reagents as follows

Reagents	*B*	*S*	*C*	*T*	*Ts*
Buffered Substrate	—	—	1 ml	1 ml	1 ml
Distilled water	2.2 ml	1.2 ml	1 ml	1 ml	1 ml
Mix well and incubate for 3 min. at 37°C					
Working Std.	—	1 ml	—	—	—
1 M tartarate	—	—	—	—	0.05 ml
Serum	—	—	—	0.2ml	0.2 ml
Mix well and incubate for 1hr. at 37°C					
0.5 N NaOH	1 ml	1 ml	1 ml	1 ml	1 ml
Serum	—	—	0.2 ml	—	—
0.5 N $NaHCO_3$	1 ml	1 ml	1 ml	1 ml	1 ml
4- aminoantipyrine	1 ml	1 ml	1 ml	1 ml	1 ml
Pot. Ferricyanide	1 ml	1 ml	1 ml	1 ml	1 ml

Mix well after addition of each reagent and measure the OD of blank, std., test, control and tartarate stable against distilled water at 510 nm.

Calculations

$$\text{Serum acid phosphatase activit in KA units} = \frac{\text{OD of Test} - \text{OD of control}}{\text{OD of std.} - \text{OD of blank}} \times 5$$

$$\text{Prostatic fraction in KA units} = \frac{\text{OD of Test} - \text{OD of Ts}}{\text{OD of std.} - \text{OD of B}} \times 5$$

Normal values	*Males*	*Females*
Total	2.5 to 11 U/L	0.3 to 9U/L
Prostatic	0.2 to 3.5 U/L	0.0 to 0.8 U/L

Clinical Significance

Determination of serum ACP is important in detecting and monitoring carcinoma of the prostate, in prostatic carcinoma with metastasis, total ACP activity may reach 40 to 50 times the upper limit. Moderate elevations in total ACP activity often occur in Paget's disease, hyperparthyroidism and breast cancer. Increased serum ACP levels are also seen Gaucher's disease and in myelocytic leukemia. As it is present in high concentration in semen, it is utilized in forensic medicine in the investigation of rape offences.

21 Body Fluids

SEMINAL FLUID

Physiology

It has four fractions—testes, epididymis, seminal vesicle and the bulbourethral glands. The spermatozoa are produced in the seminiferous tubule.

Prostate fluid is a high concentration of acid phosphatase, citric acid and enzymes responsible for coagulation and liquefaction.

Testing

It is needed in case of infertility, post-vasectomy, forensic analysis and in artificial insemination programs.

Collection and Handling

Abstinence 3 days is needed. Use sterile container. It should be delivered within one hour. Record collection and receipt time. Maintain standard precautions.

Analysis

1. Macroscopic: Appearance, volume, viscosity and pH
2. Microscopic: Count, motility and morphology
3. Normal values:
   a. Color – Translucent gray white color
   b. Volume – 2–5 ml
   c. Viscosity – Pours in droplets
   d. pH – 7.2 – 8.0

e. Count – 20 – 160 million/ml
f. Motility – >50% within one hour
g. Morphology – >30% normal forms (strict)
h. WBC – <1.0 million/ml

Additional Testing

Sperm viability, seminal fluid fructose, anti-sperm antibodies, microbial testing, chemical testing, post-vasectomy analysis and sperm function tests.

AMNIOTIC FLUID

Physiology

Present in the amnion, sac surrounding the fetus. Needed for fetal maturation. Its composition is like maternal plasma.

Testing

It is needed in case of suspected chromosomal abnormalities, metabolic disorders, neural tube defects, HDN -Hemolytic disease of the newborn, gestational age, infection and fetal maturity.

Collection and Handling

Collection by amniocentesis – needle aspiration.

30 ml of fluid is collected (maximum amount). Protect it from light – collect in amber-colored tubes. It must be delivered to laboratory promptly. Procedures should be performed immediately.

Filtration or centrifugation important. Standard precautions should be taken.

Analysis

Physical - Color and appearance

Tests for fetal distress: Hemolytic disease of the newborn (HDN) and neural tube defects

Tests for fetal maturity: Fetal lung maturity, Lecithin – sphingomyelin ratio, amniostat –FLM, foam stability, microviscosity and lamellar bodies and optical density.

CEREBROSPINAL FLUID

It is a clear fluid, circulates in the brain and spinal cord. Normal adult has from 90–150 ml and newborn 10–60 ml of CSF.

Function

It acts as buffer, regulates intracranial pressure, carries nutrients to the nervous system and serves as excretory channel for metabolic wastes in the CNS.

Collecting a specimen: invasive test with potential harm to patient. It is done only for serious reasons like to diagnose meningitis, brain hemorrhage, and diagnosis of neurological disease or malignancy. Only a physician collects CSF as it involves risk to patient. Carefully handling of specimen in necessary. Three separate tubes of about 5 ml are collected and numbered in sequence.

It acts as blood-brain barrier. CSF is not an ultrafiltrate of plasma. Many drugs do not enter the CSF from the blood. Some electrolytes are more concentrated others are less concentrated.

Protein is found only in very small amount. Chemistry tests and microbial examination is done from the first tubes and the last one is for cell counts.

CSF samples should never be refrigerated. Certain viral studies may require immediate freezing at very low temperatures. Never discard CSF until all tests are done.

Gross appearance: Normal CSF is crystal clear and the consistency of water.

Turbidity: May indicate white cells, bacteria, excess protein or fat. Radiographic dye will give the CSF an oily look. Clotting may be from a traumatic tap.

Color: Bloody fluid — can be from a traumatic tap or may indicate subarachnoid hemorrhage xanthochromia — may indicate bleeding, lysed RBCs or high protein levels.

CSF cell counts: Normally very few cells are found in CSF— no RBCs, 0–8 WBCs

PMNs are seen in bacterial infection and lymphocytes are seen in viral infection.

The cells are usually counted manually, with undiluted specimen.

Morphologic Examination

When a cell count is over 30 white cells/microliter a differential count is done smear is made from centrifuged sediment, and stained. Cytospin yields better cell count with small amount centrifuged sediment may be used.

Chemistry tests: CSF contains same chemicals that are found as in plasma. Normal values are different because of selective filtration.

Proteins: Protein tests and electrophoresis are common tests of many disease states. Normal range is 12 - 6 mg/dl (less than 1% of plasma concentration).

Increased protein levels are seen in infections, decreased levels, and leakage of fluid from CNS.

Glucose: Usually about 60–80 percent of blood glucose, should be measured at the same time difference is significant. Bacteria utilize glucose therefore glucose level reduced in bacterial meningitis, but not in viral. Glucose is elevated in diabetic coma.

Other Chemical Tests

Glutamine: Indirect measure of waste products. It is seen in some liver disorders, bilirubin and chloride, and lactate dehydrogenase.

Microbiological examination: Gram stains and cultures are useful to diagnose acute bacterial meningitis. Organisms can be seen in the Gram stain acid fast stains for TB India ink stains for Cryptococcus.

SEROUS FLUIDS

It includes pleural, pericardial and peritoneal fluids contained within closed cavities of the body. These fluid fills space between layers of cells to lubricate the surfaces as they move against each other. The fluids are formed and reabsorbed as volume is very small. Increased volume is referred to as an effusion.

Transudates: Increase in fluid volume (effusion) occurs in many conditions. Transudate is usually the result of a systemic disease. Transudates result from abnormal movement of fluid across a membrane.

Exudates: The effusions result from an inflammatory response serous effusions are classified as transudate or exudate by protein content. Transudates usually have less than 3 g/dl exudates usually over 8 g/dl.

Collection: Strictly antiseptic conditions are needed. It is collected by aspiration. It may be collected for diagnostic purposes or to relieve excess accumulation. EDTA tube for cell counts, morphology and differential, anticoagulated sample for chemical analysis, sterile tube for Gram stain and culture are needed. Keep extra tubes for cytology tumor studies.

DESCRIPTION OF SEROUS FLUIDS

Pleural Fluid

It is about 1-10 ml moistening the pleural surfaces.
Accumulation of pleural fluid occurs in congestive heart failure or in case of decreased drainage.

Pericardial Fluid

It is present in space around the heart. Normally about 25 - 50 ml of fluid is present. It is clear, straw colored. It is formed continually and is reabsorbed by lymph vessels. Abnormal accumulation can inhibit the heart action.

Peritoneal Fluid

It is present in the abdominal and pelvic cavities. The quantity is usually less than 100 ml. The abnormal accumulation causes severe pain. Hemorrhage, ruptured organ and postoperative complications can be observed.

Examination of Serous Fluids

Gross appearance: Normally straw colored (transudate). Turbidity increases with cells and debris. Cloudy may indicate infection. Bloody- may be result of collection procedures or may mean blood in cavity.

Cell counts: Done on anticoagulated specimen by hemacytometer usually on undiluted specimen. The WBC count > 500/microliter are

significant causes inflammation. The RBC count > 10,000/ul may result in trauma.

Other examinations: Differential white cell count and microbiological Gram stain can be performed.

Chemical analysis: Protein to distinguish exudate from transudate and glucose – of which low levels could indicate bacterial infection.

SYNOVIAL FLUID

This is a fluid contained in joints. Normal synovial fluid is ultrafiltrate of plasma with extrahyaluronate and is more viscous than CSF. It lubricates the joints. It is about 1 ml in each large joint and more viscous than CSF. The WBC count is < 200 ul. No RBC or crystals are seen.

Collection and Analysis

It is usually aspirated from joint by arthrocentesis needle aspiration. It is collected in both plain and anticoagulated tubes. Use sterile tube for microbiology, EDTA for cell counts and plain tube for gross examination.

Analysis for cells and crystals, Gram stain and culture is prepared.

Swelling and pain are the complains from patients.

In case of noninflammatory joint disease seen in osteoarthritis, traumatic arthritis and in neurogenic joint disease, the fluid is clear and viscous, with low cell count and glucose and protein are normal.

In case of inflammatory joint disease, rheumatoid arthritis and lupus arthritis the fluid is cloudy, yellow with low viscosity, moderately high cell count. Glucose normal and protein high. Synovial infections includes mostly bacterial infections. The fluid is cloudy, may be yellow or green and milky with low viscosity, very high cell count, low glucose and high protein.

Crystal induced effusion is seen in gout and pseudogout. Here, the fluid is yellow, cell count may be increased, crystals seen. Monosodium urates seen in gout and calcium pyrophosphate dihydrate in pseudogout.

Hemorrhagic effusions is presence of red blood cells in traumatic injury, coagulation deficiencies and anticoagulation therapy.

22 Automation in Clinical Biochemistry

The trend of using auto-analyzers in laboratory is increasing day by day. An *automated analyzer* is a laboratory machine designed to measure different chemicals in a number of biological samples quickly, with minimal human assistance.

The chemicals and other qualities of blood and other fluid measured may be useful in the diagnosis of disease.

Different methods of putting samples through have been invented, but usually involve placing test tubes of sample into racks, which can be moved along a track, or circular carousels that rotate to make the sample available. To protect the health and safety of laboratory staff many analysers feature closed tube sampling to prevent workers from direct exposure to samples.

Samples can be processed in batches, or continuously.

ROUTINE BIOCHEMISTRY ANALYZERS

These are machines that process the bulk of the samples going into a hospital or private medical laboratory. And the results should be out as quickly as possible. There will often be a method that can get urgent specimens moved more quickly through.

The types of tests required are often enzyme levels (such as many of the liver function tests), ion levels (e.g. sodium and potassium), and other chemicals (such as albumin orcreatine).

Simple ions are done with ion selective electrodes, that let one type of ion through, and measure voltage differences. Enzymes are measured by the rate they change one coloured substance to another; the results for enzymes are given as an activity, not a concentration of enzyme. Other tests use colorimetric changes to determine the concentration.

Turbidity (as created when an antibody reacts with a test compound) can also be measured with these machines.

Examples of these types of machines are:

TECHNICON AUTO ANALYZER

AutoAnalyzer is an automated analyzer using a special flow technique named "continuous flow analysis (CFA)" first made by the Technicon corporation. The instrument was invented 1957 by Leonard Skeggs, and commercialized by Jack Whitehead's Technicon Corporation. The first applications were for clinical analysis, but methods for industrial analysis soon followed.

Instruments

The best known of Technicon's CFA machines are the AutoAnalyzer II (introduced 1970), the Sequential Multiple Analyzer (SMA, 1969), and the Sequential Multiple Analyzer with Computer (SMAC, 1974). Bran+Luebbe continued to manufacture the AutoAnalyzer II and TRAACS, a micro-flow analyzer for environmental and other samples, and went on to develop the AutoAnalyzer 3 in 1997 and the QuAAtro in 2004.

Today there are other manufacturers of CFA instruments. Astoria-Pacific International, for example, was founded in 1990 by Raymond Pavitt. Its products include the Astoria Analyzer lines for Environmental and Industrial applications; the SPOTCHECK Analyzer for Neonatal screening; and FASPac (Flow Analysis Software Package) for data acquisition and computer interface.

Clinical Analysis

AutoAnalyzers were used mainly for routine repetitive medical laboratory analyses, but they had been replaced during the last years more and more by discrete working systems which allow lower reagent consumption. These machines typically determine levels of albumin, alkaline phosphatase, Serum Glutamic Oxaloacetic Transaminase (SGOT), blood urea nitrogen, bilirubin, calcium, cholesterol, creatinine, glucose, inorganic phosphorus, proteins, and uric acid in

blood serum or other bodily samples. AutoAnalyzers automate repetitive sample analysis steps which would otherwise be done manually by a technician, for such medical tests as the ones mentioned previously. This way, an AutoAnalyzer can analyze hundreds of samples every day with one operating technician. Early AutoAnalyzer instruments each tested multiple samples sequentially for individual analytes. Later model AutoAnalyzers such as the SMAC tested for multiple analytes simultaneously in the samples.

Operating Principle

In a continuous flow analyzer, a peristaltic pump contains several tubes including one for the sample, one or more for various reagents and one or more to generate air bubbles. The pump tubes deliver into the "manifold" of junctions, coils and tubing where the reactions take place. In Segmented Flow Analyzers (SFA), the sample is mixed with small reproducible volumes of the required reagents and air bubbles are introduced into the flow, creating about 20 – 100 segments of liquid for each sample, keeping them separated as they flow sequentially through the tubing. The inlet side of the sample pump tube is connected to the sample probe in an autosampler. The sample probe moves between the small cups holding liquid samples and a reservoir of wash solution, normally pure water, which also serves to generate a baseline response. The sample / reagent mixture flows through mixing coils, and depending on the method a heated coil for elevated reaction temperature or other modules to develop a color proportional to the amount of analyte in each sample. The samples with developed color flow through a colorimeter to measure the color. Other detectors such as a flame photometer, a fluorometer or an ISE module are used for some applications.

Flow Injection Analyzers (FIA) operate similarly to SFA, but without the air segmentation. A flow injection analyzer introduces sample into a flowing stream of reagents using an injection valve. The reagents and sample are mixed together while passing through tightly coiled narrow bore teflon tubing and other modules to develop a product that is measurable by a detector. Normally the same chemistries possible by SFA are possible by FIA. SFA methods have a clear advantage at obtaining lower detection limits, and the ability to bring all chemical reactions to completion prior to measurement.

Previously a chart recorder and more recently a PC records the detector output as a function of time so that each sample output appears as a peak whose height depends on the analyze level in the sample. In medical testing applications and industrial samples with high concentrations or interfering material, there is often a dialyzer module in the instrument in which the analyte permeates through the diaphragm into a separate flow path going on to further analysis. The purpose of a dialyzer is to separate the analyte from interfering substances such as protein, whose large molecules do not go through the dialysis membrane but go to a separate waste stream. The reagents, sample and reagent volumes, flow rates, and other aspects of the instrument analysis depend on which analyte is being measured.

HITACHI 917 (FIG. 22.1)

The *Hitachi 917* (Fig. 22.1) is an automated biochemistry analyzer used by medical laboratories process biological fluid specimens, such as urine, cerebrospinal fluid but most commonly, blood.

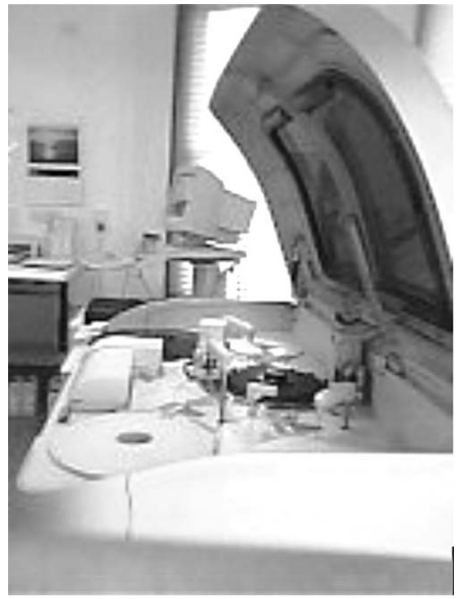


Fig. 22.1: Hitachi 917
(For colour version see Plate 3)

Manufactured by Boehringer Mannheim, the Hitachi 917 is a commonly used routine chemical bichromatic analyzer. Capable of doing 1200 test/hour with ISE, it is a popular choice among small to medium size laboratories.

Appearance and Use

The 917 has two trays for racks, plus a stst rack. Racks that hold five teat tubes slide in on the left side of the machine. There are two reagents carousels on the right side of the 917. In the centre, towards the back, are the reaction vessels, where the chemical reactions take place.

Ion-selective electrode (ISE) reagents and components are in front of the reaction carousel.

Tests Available

- Ion-selective electrode – Na, K, Cl
- Rate – CK
- End point
- Immunoturbidity

COBAS MIRA

The Roche Cobas Mira is a benchtop, random access biochemistry analyser. This system allows for the selective analysis of chemistries in either a routine or STAT mode. Testing sequence is by patient rather than test (batch). The optional ISE module for sodium, potassium and chloride determinations expand the capabilities of the instrument.

The Roche Cobas Mira is ideal in the laboratory situation where patient samples arrive throughout the day and night. With random access instrumentation, these samples may be placed on the analyzer and results will be available within minutes. With random access, samples do not have to be saved for a major run as with batch analyzers. STAT samples may be placed on the instrument at any time. Once the STAT has been completed, the Cobas Mira automatically returns to processing the routine samples. Communication with the Roche Cobas Mira is done via the integrated control panel. Through the control panel the operator selects tests, reviews results and programs tests, profiles and system parameters.

Other features of the Roche Cobas Mira include; a throughput of 140 tests/hr, microprocessor controlled XYZ pipetting system, 104 test channels including 23 preprogrammed methods and the capacity to hold 2100 patient results, capacity for 72 cuvettes (six 12 cuvette segments), cuvette volume of 150-600 µL, and an analysis interval of 25 seconds. The system also includes a security system for laboratory-defined parameters.

The instrument checks for the integrity of the cuvette, the rack reader, temperature, the monitor and printer.

The other important examples include :

1. Hitachi 912
2. Abbott Aeroset
3. Dade Dimension
4. Beckman-Coulter LX
5. Berkman Astra
6. Dupont Automated Clinical Analyzer
7. Kodak Ektachem 700

IMMUNO-BASED ANALYZERS

Because many substances (such as hormones or drugs) have no colour, and cannot cause another substance to change colour, antibodies must be used to detect them.

The concentration of these compounds is often too low to cause a measurable increase in turbidity when bound to antibody, so other, more specialised, methods must be used.

HAEMATOLOGY ANALYSERS

These are used to perform full blood counts, erythrocyte sedimentation rates (ESRs) or coagulation tests.

Cell Counters

Cell counting machines sample the blood, and analyse its cell populations using both electrical and optical techniques. Electrical analysis involves passing a dilute solution of the blood through an aperture across which an electrical current is flowing. The passage of

cells through the current changes the electrical impedance between the terminals (the Coulter principle). A lytic reagent is added to the blood solution to selectively burst the red cells, leaving only white cells and platelets intact, then the solution is passed through a second detector. This allows the a differential count of RBCs against WBCs and platelets to be obtained. The Platelet count is easily separated from the WBC count by the smaller impedance spikes they produce in the detector due to their small volume.

Optical detection is utilised to gain a differential count of the populations of white cell types. A dilute suspension of cells is passed through a flow cell, which passes cells one at a time through a capillary tube past a laser beam. The reflectance, transmission and scattering of light from each cell is analysed by sophisticated software giving a numerical representation of the likely overall distribution of cell populations.

Reticulocyte counts can be performed by many analysers, but are just as often carried out manually by staining blood in a Merrett tube and performing a cell count under the microscope. Some analysers have a modular slide maker which is able to both produce a blood film of consistent quality and stain the film.

Examples of full blood count machines are:

- Abbott Cell-Dyn product line
- Sysmex XE 2100
- Beckman-Coulter Gen-S article

Coagulometers

Automated coagulation machines or Coagulometers measure the ability of blood to clot by performing several types of tests including clotting screens, INRs, lupus anticoagulant screens and factor assays.

Coagulometers require blood samples that have been taken in tubes containing trisodium citrate as an anticoagulant. These are used rather than EDTA anticoagulated tubes as the mechanism behind the anticoagulant effect is reversible by the addition of calcium ions to the solution. Depending on the test different substances can be added to the blood plasma to trigger a clotting reaction. The progress of clotting is measured optically by measuring the absorbance of a

particular wavelength of light by the sample and how it changes over time.

Coagulation machines include:

- Sysmex CA 1500
- Biomerius MDA

Other Haematology Apparatus

Automatic ESR readers, while not strictly analysers, hold a rack of samples for an hour, then after an hour determine how far the red cells have fallen, by detecting levels with light beams.

As ESR tests become less popular they are being replaced by plasma viscosity tests. The advantage of this test over ESR is that less variables can affect the result so it can give a more direct impression of the plasma protein content of a sample. The analysers commonly work by drawing a small sample of plasma through a narrow capillary using a constant pressure and measuring the time taken for the sample to move a known distance.

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